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EFECTO DE LA EXPOSICIÓN NEONATAL A BISFENOL A (BPA) SOBRE LA RESPUESTA INMUNE AL CÁNCER DE MAMA EN UN MODELO MURINO

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A mi padre, mi madre y mi hermana, mis primeros grandes amores.

A la persona que ilumina mis días.

A esa fuerza que, en contra de la entropía, logra ser fuente de orden, de belleza y de vida.

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Índice

Resumen

El Bisfenol-A (BPA) es un compuesto disruptor endócrino de carácter estrogénico. La exposición al BPA durante etapas tempranas del desarrollo puede derivar en efectos a largo plazo. Dado que los sistemas inmunológico y endócrino interactúan bidireccionalmente, la disrupción endócrina puede causar alteraciones en el sistema inmunológico, incluida la respuesta antitumoral. En este trabajo estudiamos las potenciales alteraciones en la respuesta antitumoral en la vida adulta tras una exposición neonatal a BPA.

Ratones neonatos (3er día postnatal) de la cepa singénica BALB/c fueron expuestos a una dosis única de 250 µg/kg de BPA. Una vez que se alcanzó la plena madurez sexual, se indujo la formación de un tumor mamario al inyectar células de la línea 4T1, la cual deriva de un adenocarcinoma mamario espontáneo de ratón BALB/c y por lo tanto permite el uso de individuos compatibles e inmunocompetentes.

Transcurridos 25 días desde la inducción, los ratones del grupo expuesto a BPA desarrollaron tumores de mayor tamaño (Peso normalizado contra control = 1.88; intervalo de confianza 95% (I.C.): 1.48, 2.28; p= 0.007).

Para evaluar la respuesta inmune antitumoral a nivel local, se realizó un análisis del infiltrado leucocitario de la masa tumoral mediante citometría de flujo e inmunofluorescencia. Asimismo, se analizaron las subpoblaciones celulares presentes en los nódulos linfáticos periféricos drenantes y el bazo, para evaluar la respuesta a nivel regional y sistémico, respectivamente. Se observó que el microambiente tumoral de los individuos expuestos a BPA presentaba un mayor porcentaje de linfocitos T reguladores (42.8%, I.C. 30.3%, 55.3% vs 21.5%, I.C. 14.1%, 28.9% en control intacto; p= 0.0031).

Se analizó por RT-PCR la expresión de las citocinas proinflamatorias en el microambiente tumoral y se observó que las citocinas proinflamatorias TNF-α e IFN-γ presentaban un menor nivel de expresión en el grupo expuesto a BPA. Adicionalmente, por análisis de RT-PCR se evaluó el perfil de activación de los macrófagos asociados a tumor, observándose que presentaban un perfil de activación alternativa en todos los

grupos experimentales. Sin embargo, el grupo expuesto a BPA mostró una menor expresión del marcador Fizz-1.

Dado el carácter estrogénico del BPA, se evaluó la expresión del receptor de estrógenos ERα en los linfocitos T, B, células NK y macrófagos, hallándose diferencias en la expresión de este receptor en los linfocitos T totales, macrófagos y células NK asociados tanto a la exposición al BPA como al desarrollo tumoral.

En conclusión, la exposición neonatal a BPA modifica la respuesta antitumoral en la vida adulta, siendo uno de los probables mecanismos la alteración de los patrones de expresión de ERα en las células del sistema inmune.

Estos hallazgos muestran un nuevo aspecto en el cual la exposición a BPA puede contribuir al desarrollo y progresión del cáncer de mama: la modulación de la respuesta inmune antitumoral.

Abstract

BPA is an oestrogenic endocrine disrupting chemical (EDC) compound. Exposure to BPA in as early as pregnancy leads to lifelong effects. Since endocrine and immune systems interact in a bidirectional manner, endocrine disruption may cause permanent alterations of the immune system, affecting a future anti-tumoral response. In this work, we studied the potential alterations in anti-tumoural immune response in adult life, after a neonatal exposure to BPA.

Neonate (PND 3) female syngeneic BALB/c mice were exposed to a single dose of 250 µg/kg BPA. Once sexual maturity was reached, a mammary tumour was induced injecting 4T1 cells *in situ*, these cells are derived from a spontaneous adenocarcinoma in a BALB/c mouse and therefore allows for an immunocompetent recipient.

After 25 days of injection, showing no major endocrine alterations, BPA-exposed mice developed larger tumours (Relative weight compared to unexposed control = 1.88; 95% confidence interval (CI): 1.48, 2.28; p= 0.007).

As a mean to study local anti-tumoural immune response, tumour leukocytic infiltrate analysis was performed by flow cytometry and immunofluorescence, revealing a higher proportion of regulatory T lymphocytes in the BPA-exposed group (42.8%, CI 30.3%, 55.3% vs 21.5%, CI 14.1%, 28.9% in unexposed control; p= 0.0031).

RT-PCR analysis of tumour samples showed a decreased expression of proinflammatory cytokines TNF-α and IFN-γ. Moreover, RT-PCR analysis of activation profile of TAMs showed that, while all samples displayed an alternative activation profile, the TAMs from BPA-exposed group had a lower expression of Fizz-1.

Since BPA is an oestrogenic EDC, ERα expression was assessed by flow cytometry in T lymphocytes, B lymphocytes, NK cells and macrophages, revealing differences associated both to BPA exposure and tumour development.

In conclusion, neonatal exposure to BPA modifies anti-tumoural immune response in adulthood, probably by altering ERα expression patterns in cells of the immune system.

These findings show a new aspect whereby early life BPA exposure can contribute to breast cancer development and progression by modulating the anti-tumoral immune response.

Lista de abreviaturas

AR	Receptor de andrógenos
Arg-1	Arginasa
BPA	Bisfenol A
CDE	Compuesto disruptor endócrino
CPAs	Células presentadoras de antígeno
DES	Dietilestilbestrol
DPN	Día postnatal
ERα	Receptor de estrógenos alfa
ERβ	Receptor de estrógenos beta
FasL	Ligando de Fas
Fizz-1	Found in inflammatory zone
FoxP3	Forkhead Box P3
I.C.	Intervalo de confianza
IFN-γ	Interferón gamma
IL-	Interleucina
iNOS	Sintasa de óxido nítrico inducible
LPS	Lipopolisacárido
LTCs	Linfocitos T citotóxicos
M1	Macrófago clásicamente activado
M2	Macrófago alternativamente activado
MATs	Macrófagos asociados a tumor

MHC	Complejo principal de histocompatibilidad
NK	Natural killer
PR	Receptor de progesterona
RT-PCR	Reacción en cadena de la polimerasa previa retrotranscripción de RNA
SERM	Modulador selectivo del receptor de estrógenos
T helper	Linfocitos T cooperadores
TGF-β	Factor transformante del crecimiento beta
TNF-α	Factor de necrosis tumoral alfa
TRAIL	Ligando inductor de apoptosis relacionado con TNF
Treg	Linfocitos T reguladores
Ym-1	Proteína similar a quitinasa – 3

1. Introducción

1.1 Cáncer de mama

El cáncer de mama es un conjunto de enfermedades caracterizado por la proliferación de células anormales (transformadas) que deriva en un crecimiento descontrolado del tejido mamario.

1.1.1 Anatomía de la glándula mamaria

La mama se encuentra localizada sobre la pared torácica. La porción glandular está situada en el centro, rodeada y atravesada por tejido adiposo; la grasa proporciona a la mama su consistencia blanda. La glándula se compone de 12 a 20 lóbulos, los cuales se dividen en lobulillos y éstos a su vez en alvéolos secretorios. Los lóbulos desembocan en los ductos, los cuales convergen hasta un sitio debajo del pezón en forma de conductos lactíferos. La mama cuenta con unos ligamentos que penetran desde la piel hasta la aponeurosis pectoral, proporcionando un armazón de bandas de tejido fibroso que sostienen a los lobulillos y lóbulos [1].

1.1.2 Tumores mamarios

Los tumores mamarios son el resultado del crecimiento desmesurado de las células de algún tejido mamario. A los tumores derivados del epitelio mamario se les denomina genéricamente carcinomas y a aquéllos derivados del mesénquima, sarcomas.

Dentro de los carcinomas se encuentran dos grandes grupos: el ductal, que se origina en los conductos mamarios y el lobular, que tiene origen en los lóbulos. En ambos casos, el tumor puede encontrarse localizado, en cuyo caso se le denomina carcinoma *in situ* o, por el contrario, puede encontrarse diseminado, en cuyo caso se denomina carcinoma infiltrante [1].

Los tumores de origen mesenquimatoso, que es parte del tejido conjuntivo, ocupan aproximadamente el 1% de todos los tumores mamarios malignos. Entre ellos, el más frecuente es el fibrosarcoma, el cual se caracteriza por un crecimiento rápido [1].

1.1.3 Estadísticas

El cáncer de mama se encuentra entre los tipos de cáncer más frecuentes. A nivel mundial, de acuerdo al último reporte de Globocan [2], en 2012 ocupó el primer lugar en prevalencia a 5 años en población adulta de ambos sexos, seguido del cáncer de próstata y colorrectal.

En México, en 2014, el INEGI [3] reportó que del total de casos de cáncer diagnosticados en la población de 20 años y más, el cáncer de mama es el de mayor presencia, con 19.4% del total, representando el 30.9% de los casos de cáncer en mujeres. En 2015, el mismo instituto reportó que la incidencia de este cáncer entre la población de 20 años y más fue de 14.8 casos nuevos por cada 100,000 personas, que equivaldría a 11,250 casos totales considerando el total poblacional en ese rango de edad.

Es importante mencionar que también en 2014 se observó un incremento en la tasa de mortalidad por tumor maligno de mama, en comparación con años previos, siendo de 15.21 defunciones por cada 100,000 habitantes. Este dato continuó con la tendencia en ascenso de la mortalidad de este padecimiento, desde el año 2007 [3], lo cual llama a la reflexión, dados los constantes esfuerzos dirigidos a su tratamiento y detección precoz.

1.2 Inmunopatología del cáncer

La intervención del sistema inmunológico en el cáncer comienza con la detección de las células transformadas y no con el ataque y contención del desarrollo de una masa tumoral establecida. En la década de los 50s, Burnett propuso la teoría de la *Inmunovigilancia* [4], en la cual las células del sistema inmunológico innato detectan a las células transformadas, las atacan y despiertan una respuesta del sistema inmunológico adaptativo para eliminarlas, en cuyo defecto se genera una masa tumoral y se inicia el cáncer.

1.2.1 Inmunovigilancia, Inmunoedición e inmunosubversión

El proceso anteriormente descrito es más complejo de lo que originalmente se creía y comprende no sólo una serie simple de eventos, sino procesos y etapas alternativas, tales como la *Inmunoedición*, y la *Inmunosubversión* [5,6].

A pesar de que, en un principio, durante la *inmunovigilancia* se detecte y se genere una respuesta inmunológica robusta contra las células transformadas, no todas son eliminadas, existiendo células que logran evadir el reconocimiento y ataque del sistema inmunológico. Estas células proliferan y, en respuesta, se generan nuevos mecanismos del sistema inmunológico (tales como la respuesta humoral) que imponen una nueva presión de selección inmunológica sobre las células transformadas.

Durante este proceso, denominado *Inmunoedición*, se generan clonas de células transformadas con menor inmunogenicidad y con mayor resistencia a los mecanismos citotóxicos del sistema inmunológico. Otra característica es que estas clonas tienen mayores propiedades inmuno-moduladoras, tales como una mayor habilidad para producir y secretar factores solubles que modifican el microambiente tumoral de manera que afecta las funciones efectoras de las células del sistema inmunológico e induce tolerancia inmunológica y anergia en ciertos tipos celulares.

En este proceso, llamado *inmunosubversión*, intervienen los linfocitos T reguladores (Treg), junto con diversos elementos en el microambiente tumoral, convirtiendo esta etapa en la estrategia clave mediante la cual las células exitosamente *editadas* escapan a la presión del sistema inmunológico[5–7].

A continuación, se describe brevemente el papel que juegan distintas poblaciones celulares del sistema inmunológico en estos procesos, que pueden observarse de manera resumida en la **figura 1**.

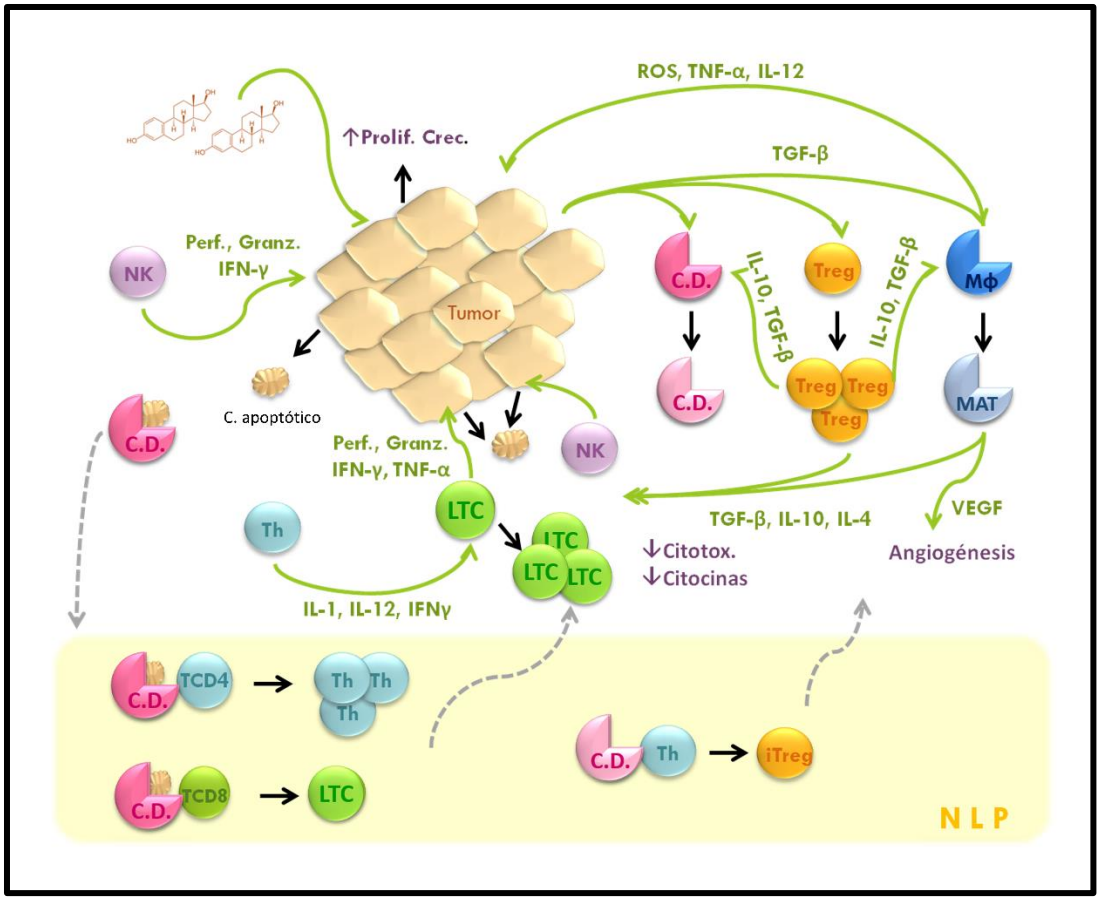

Figura 1. *Inmunovigilancia* e *inmunosubversión*. Los elementos de la inmunidad innata son los primeros en intervenir; las células NK detectan células anormales o estresadas y las atacan principalmente secretando perforina y granzimas, produciendo la apoptosis de las células tumorales. Los cuerpos apoptóticos son fagocitados y procesados por las células presentadoras de antígeno (CPAs), ya sean macrófagos (Mφ) o células dendríticas (C.D.), las cuales migran hacia los nódulos linfáticos periféricos (NLP) y presentan antígenos a los linfocitos. Posteriormente, las clonas de linfocitos T helper (Th) y T citotóxicos (LTCs) que fueron activados proliferan y orquestan la inmunidad adaptativa antitumoral. Por su parte, las células tumorales modifican el microambiente tumoral produciendo factores inmunomoduladores tales como el TGF-β, el cual disminuye la actividad de las células citotóxicas y las CPAs (con lo que se favorece la generación de linfocitos T reguladores inducidos (iTreg), se promueve la proliferación de los Treg ya existentes y modifica el perfil de activación de los macrófagos asociados a tumor (MATs) hacia un fenotipo protumoral, generando más moléculas inmunomoduladoras y factores angiogénicos [8].

1.2.1.1 Células NK

Las células NK (del inglés, Natural killer) son células efectoras del sistema inmunológico innato que controlan varios tipos de tumores, virus y patógenos microbianos limitando su crecimiento y diseminación, principalmente mediante su actividad citolítica [9]**.** Estas células son uno de los principales elementos que orquestan el proceso de *inmunovigilancia*, pues se ha asociado su baja actividad con un mayor riesgo de cáncer [10]. Las células NK *vigilan* constantemente las células del organismo sensando sus moléculas de superficie para determinar si son propias y se encuentran sanas. Para ello, las células NK expresan múltiples receptores de inhibición que tienen afinidad por las moléculas del complejo principal de histocompatibilidad (MHC) de clase I, las cuales son altamente polimórficas incluso entre individuos de la misma especie y cuya expresión puede verse alterada ante fenómenos de estrés y algunas patologías [11,12]. Por otra parte, estas células también expresan receptores de activación, cuyos ligandos rara vez se expresan en células normales, pero se expresan en respuesta al estrés y la transformación celular.

Una vez activadas, las células NK utilizan dos principales mecanismos efectores: la exocitosis de gránulos que contienen perforina y granzimas A/B y la señalización por medio de ligandos receptores de muerte celular, tales como FasL (Ligando de Fas) y TRAIL (Ligando Inductor de Apoptosis Relacionado con TNF). Adicionalmente, las células NK activadas producen citocinas que controlan el crecimiento tumoral y promueven la activación de otros tipos celulares, como el interferón gamma (IFN-γ) y el factor de necrosis tumoral alfa (TNF-α).

Por su parte, las células tumorales utilizan diversos mecanismos para resistir y evadir a las células NK, incluyendo su inhibición mediante interacciones por contacto, la secreción de factores inhibitorios como el factor transformante del crecimiento beta (TGF-β) y la modificación del microambiente tumoral, favoreciendo el reclutamiento y diferenciación de linfocitos T reguladores y favoreciendo el crecimiento de las células tumorales [13].

1.2.1.2 Macrófagos asociados a tumor

Los macrófagos son células fagocíticas residentes en casi todos los tejidos. Ante eventos como daño tisular e infección, los monocitos circulantes son reclutados hacia el sitio del evento y se diferencian a macrófagos tisulares. Los macrófagos expresan una variedad de receptores de superficie que les permiten reconocer moléculas características en patógenos, células estresadas o necróticas, cuerpos apoptóticos y restos celulares. Una vez que entran en contacto con estos elementos, los macrófagos los fagocitan y los procesan.

Los macrófagos, así como las células dendríticas, son considerados células presentadoras de antígeno profesionales (CPAs) y constituyen el vínculo entre la inmunidad innata y la inmunidad adaptativa. Después de que un patógeno o una célula dañada es fagocitada y procesada, el macrófago migra hacia el nódulo linfático más próximo y en el transcurso madura, adquiriendo la expresión de moléculas coestimuladoras de superficie (CD40, CD80, CD86), las cuales le permiten activar a linfocitos T.

Si bien actualmente se sabe que los macrófagos pueden presentar toda una gama de perfiles de activación [14], en términos generales es posible hablar de dos clases de macrófagos: clásicamente activados (M1) y alternativamente activados (M2). Los macrófagos M1 se generan tras la estimulación con IFN-γ solo o en conjunto con moléculas bacterianas tales como el lipopolisacárido (LPS). Por otra parte, los macrófagos M2 son polarizados por distintos estímulos, como las citocinas interleucina (IL-) 4, IL-13, IL-10, TGF-β y glucocorticoides[14,15].

En cuanto a su función efectora, en términos generales los macrófagos M1 combaten microorganismos y producen grandes cantidades de citocinas proinflamatorias, mientras que los macrófagos M2 modulan la respuesta inflamatoria, promueven la angiogénesis y la remodelación y reparación de tejidos. Sin embargo, es importante destacar que los estados de polarización M1 y M2 son los extremos de un amplio rango de estados funcionales [14].

Los macrófagos asociados a tumor (MATs) suelen presentar un fenotipo similar al M2 [14,16,17], el cual está caracterizado por la expresión de Arg-1, Fizz-1 y Ym-1, entre otros y una mínima expresión de iNOS. Sin embargo, el fenotipo funcional de los MATs no es homogéneo entre los diversos tipos y modelos de cáncer y se ha planteado que incluso puede ser variable dependiendo de la localización dentro del tumor [18].

En la mayoría de los tipos de cáncer, incluido el cáncer de mama, los MATs son antiinflamatorios y se correlacionan con un mal pronóstico [19]. Esto se debe a que los MATs no son CPAs competentes, ya que la expresión de moléculas coestimuladoras se ve disminuida, a tal grado que, en vez de activar a linfocitos T, ocasionan la anergia de estas células.

Por otra parte, estos macrófagos producen IL-10, la cual es una citocina inmunomoduladora que disminuye la activación de linfocitos T cooperadores, citotóxicos y células NK, entre otros. Además, la participación de los MATs no se limita a la inmunomodulación en el microambiente tumoral, pues contribuyen de manera directa a la vascularización de mismo, produciendo factores angiogénicos como el factor de crecimiento vascular-endotelial (VEGF), además de que favorecen la invasividad al secretar metaloproteasas que degradan la matriz extracelular [20].

1.2.1.3 Linfocitos T citotóxicos

Los linfocitos T citotóxicos (LTCs), caracterizados por la expresión del correceptor CD8, son activados por las CPAs en los nódulos linfáticos, tras lo cual experimentan una expansión clonal, adquieren su fenotipo efector y migran hacia el sitio en el que ha ocurrido el evento que activó a las CPAs. Una vez que los LTCs encuentran alguna célula que expresa el antígeno contra el cual están dirigidos inducen la muerte de la misma por contacto, mediante TRAIL o mediante la secreción de perforina y granzimas. Adicionalmente, los LTCs secretan IFN-γ y TNF-α [21]. El IFN-γ contribuye a la activación de los macrófagos y células dendríticas, aumentando su capacidad fagocítica, además de favorecer la diferenciación de los linfocitos T helper hacia un fenotipo Th1. Por su parte, el TNF-α es considerado un inhibidor de la tumorigénesis pues induce la muerte

por apoptosis de células transformadas, además de ser una de las citocinas clave para la respuesta inflamatoria.

En cuanto a la respuesta inmune antitumoral, los LTCs son capaces de infiltrar y eliminar a las células transformadas durante etapas tempranas del desarrollo tumoral, pero pierden su capacidad citotóxica ante la exposición crónica al microambiente tumoral. Pese a lo anterior, se sabe que estos linfocitos aún son capaces de producir IL-2 e IFN-γ.

1.2.1.4 Linfocitos T cooperadores

Los linfocitos T cooperadores (T helper, en inglés), caracterizados por la expresión del correceptor CD4, se encuentran tanto en circulación como residiendo en nódulos linfáticos y órganos linfoides en su estado virgen. Cuando estos linfocitos son activados por las CPAs, proliferan y adquieren su carácter efector, dependiendo del microambiente en el que se encuentren. Una vez activados, los T helper migran hacia el sitio del evento que inició la respuesta inmune, en donde producen citocinas y quimiocinas que modulan y orquestan esta respuesta.

Existe una variedad de linfocitos T helper, dependiendo del perfil de citocinas que producen. Entre los más representativos, se pueden mencionar a los T helper (Th-) 1, Th2 y Th17. Los Th1 se caracterizan por secretar IFN-γ y TNF-α. Los Th2 producen IL-4, IL-5 e IL-13. Los Th17 secretan IL-17A, IL-17F, IL-6, IL-22 y TNF-α.

Se ha reportado que los Th1 parecen favorecer la respuesta inmune antitumoral, al secretar citocinas proinflamatorias que ejercen un efecto directo sobre las células tumorales y sobre otras poblaciones en el microambiente tumoral. En este sentido el IFN-γ, la principal citocina Th1 juega un papel importante al ser antiproliferativa y antiangiogénica, a la vez que estimula a las células NK y a los LTCs, incrementando su capacidad citotóxica, mientras que activa a macrófagos y a células dendríticas. Por su parte, el TNF-α también posee propiedades antiproliferativas e incrementa la expresión de moléculas del MHC clase I tanto en las células tumorales como en las CPAs,

facilitando la presentación de antígeno y la susceptibilidad de las células tumorales a los mecanismos citotóxicos [22].

1.2.1.5 Linfocitos T reguladores

Los linfocitos T reguladores (Treg) constituyen entre el 5 y el 10% de los linfocitos T $CD4^+$ en la periferia y modulan la respuesta inmune mediante mecanismos de supresión [23]. Los Treg se caracterizan por la expresión del factor de transcripción FoxP3 y una elevada expresión del receptor de IL-2, CD25 [24].

Los Treg inhiben la actividad de las CPAs mediante un mecanismo dependiente de contacto que involucra las interacciones FasL/Fas y PD1/B7-H1, así como mecanismos independientes de contacto mediados por IL-10, TGF-β, IL-27 e IL-35 [23]. Asimismo, dada la elevada expresión de CD25, estos linfocitos consumen altas cantidades de IL-2, lo que conduce a una privación de esta citocina, la cual deja de estar disponible para otras poblaciones, como los LTCs y los T helper, reduciendo su potencial de activación.

De manera general, los Treg promueven el desarrollo del cáncer al suprimir la respuesta inmune antitumoral. De hecho, la eliminación de estos linfocitos por medios químicos o mediante terapia de anticuerpos restaura la respuesta inmune antitumoral en varios modelos animales, lo que deriva en la erradicación de los tumores [25].

1.3 Línea 4T1 como modelo murino de cáncer de mama

A pesar de existir una variedad de modelos animales de cáncer de mama, muchos presentan desventajas que limitan su validez como herramienta traslacional. Los primeros modelos en los que se utilizaba un xenotransplante de células provenientes de carcinomas mamarios presentaban la desventaja de la representación exclusiva de una clona celular, además de ofrecer la perspectiva de un tumor en un contexto inmunológico sumamente deprimido, lo cual no es la regla entre los pacientes. Por otra parte, las líneas

celulares murinas que pueden ser transplantadas tampoco logran representar la enfermedad, pues la metástasis que ocurre en varios de estos modelos es hematógena y casi exclusivamente pulmonar, mientras que el cáncer de mama se caracteriza por producir metástasis por vía linfática y no sólo hacia el pulmón, sino hacia el hígado y cerebro, los cuales son órganos que rara vez se ven afectados en modelos murinos que no han sido específicamente diseñados para hacer homing hacia estos sitios. Por otra parte, los modelos transgénicos en los que se sobreexpresa un oncongén resultan en tumorigénesis multifocal, lo cual no es una presentación común en la clínica [26].

La 4T1 es una línea celular derivada de un adenocarcinoma mamario de surgimiento espontáneo en un ratón BALB/c (H-2^d) por lo que no se requiere de ratones parcial o totalmente inmunodeprimidos, sino que puede introducirse en individuos inmunocompetentes de la cepa BALB/c haploidéntica. Una vez transplantadas, las células 4T1 son altamente tumorigénicas, invasivas y, a diferencia de otros modelos tumorales, tiene la capacidad de generar metástasis espontáneas en distintos órganos, incluyendo nódulos linfáticos, hígado, pulmón, cerebro y hueso [27]. Por lo tanto, los tumores generados a partir de la línea 4T1 presentan características que los hacen útiles como modelo animal del cáncer de mama humano. Y de manera particular, este modelo resulta útil para estudiar la respuesta inmune antitumoral, al desarrollarse en un contexto inmunocompetente.

Esta línea celular ha sido utilizada por algunos grupos como modelo murino de cáncer de mama triple negativo, caracterizado por la falta de expresión de los receptores de estrógenos progesterona y la no sobreexpresión del receptor Her2 [28–30]. Sin embargo, la American Type Culture Collection (ATCC) no ha evaluado la expresión de estos receptores en la línea [31] y en nuestro grupo se encontró que expresa tanto el receptor de estrógenos ERα como el receptor nuclear de progesterona (PR).

1.4 Red neuroinmunoendócrina

El sistema inmunológico interactúa bidireccionalmente con el sistema neuroendócrino, de manera que estos sistemas no constituyen entidades fisiológicas

independientes, sino que forman parte de una compleja red fisiológica, en la que las citocinas, hormonas peptídicas, hormonas esteroides y neuropéptidos regulan y modifican tanto la respuesta inmune como diversos fenómenos neuroendócrinos y, en conjunto, mantienen la homeostasis del organismo [32].

1.4.1 Modulación del sistema inmune por esteroides sexuales

En los mamíferos, el sexo se determina desde la concepción, tras la cual ocurre el desarrollo fisiológico y endócrino, generándose múltiples diferencias entre machos y hembras. Desde el periodo previo a la pubertad hasta la edad reproductiva, estas diferencias se basan en la producción, secreción y concentraciones circulantes de estrógenos, progesterona y testosterona y en la función y desarrollo del eje hipotálamo-hipofisiario-gonadal [32–35]. Además de los efectos sobre la diferenciación gonadal y demás órganos reproductivos, las hormonas sexuales determinan las diferencias sexuales en cuanto a la respuesta inmune generada ante el mismo estímulo antigénico.

Este dimorfismo se manifiesta de varias maneras. Por ejemplo, las hembras de varias especies producen mayores niveles de inmunoglobulinas y típicamente desarrollan una mayor respuesta humoral contra las infecciones. Las hembras también suelen producir más frecuentemente anticuerpos autorreactivos, lo cual se refleja en la mayor incidencia de enfermedades autoinmunes en mujeres [36,37].

Las hormonas sexuales modulan una gran variedad de fenómenos en el sistema inmunológico, incluyendo la maduración y selección de los timocitos, el tránsito celular, la proliferación de linfocitos, la expresión de moléculas del MHC de clase II y sus receptores, así como la producción de citocinas [35,38,39]. Los efectos moduladores de los esteroides sexuales están mediados por la expresión de receptores específicos para estas hormonas en las células del sistema inmunológico, siendo los receptores a estrógenos clásicos (ERα y ERβ), el receptor a progesterona (PR) y el receptor a andrógenos (AR) los más relevantes [40]

1.5 Compuestos disruptores endócrinos

La actividad humana, particularmente la industrial, ha traído consigo el incremento en gran variedad de compuestos contaminantes en el medio ambiente. Entre dichos contaminantes, se encuentran los denominados compuestos disruptores endócrinos (CDE). Los CDE han sido definidos como agentes exógenos que interfieren con la síntesis, secreción, transporte, metabolismo, mecanismo de acción o eliminación de las hormonas propias del organismo [41].

Algunos CDE pueden presentar actividad estrogénica, anti-estrogénica o anti-androgénica. Compuestos como el Dietilstilbestrol (DES), la genisteína, ftalatos, pesticidas organoclorados y los Bisfenoles (particularmente el A) son algunos de los CDE más ampliamente estudiados al respecto. Estudios epidemiológicos y experimentales, han demostrado alteraciones irreversibles en el eje reproductivo de la descendencia como consecuencia de la exposición intrauterina y/o neonatal a este tipo de compuestos en diferentes especies [42–51].

1.5.1 BPA

El Bisfenol A (BPA), es un CDE de carácter estrogénico, caracterizado por su alta afinidad a los receptores nucleares de estrógenos ERα y ERβ [52,53]. Si bien se ha reportado que la afinidad del BPA por estos receptores es baja (alrededor de 1/100 con respecto al 17β-Estradiol) [54,55], se ha identificado la unión del BPA a otros receptores como el GPR30 y el ERR-γ, a través de los cuales puede ejercer sus efectos [52,56–58]. Además, datos recientes sugieren que el BPA puede ser considerado como un modulador selectivo del receptor de estrógenos (SERM, por sus siglas en inglés), ya que este compuesto y el 17β-estradiol no siempre ejercen los mismos efectos [49].

1.5.1.1 Fuentes y niveles de exposición

El BPA es ampliamente utilizado como monómero en la elaboración de policarbonatos, resinas epóxicas –usados en la fabricación de envases de alimentos y

recubrimientos de latas, respectivamente– y selladores dentales, todos ellos materiales de uso cotidiano. Este compuesto puede liberarse de estos materiales por polimerización incompleta o hidrólisis de los enlaces éster de los polímeros que lo contienen, lo cual puede ocurrir al someterlos a altas temperaturas, condiciones ácidas o por mecanismos enzimáticos [59–62].

Los datos acerca de la exposición al BPA son controversiales, pero con base en el análisis de fluidos corporales humanos, se ha estimado un nivel sérico medio de aproximadamente 2 ng/ml [63]. Cabe resaltar que el BPA se ha detectado en diversos fluidos y tejidos, incluidos la leche materna, la placenta, el cordón umbilical y el fluido seminal [63]. Lo que se ha determinado con certeza es que la principal fuente de exposición a este CDE es la vía oral [63,64]. Tomando en cuenta los datos de liberación de BPA de productos enlatados y contenedores de alimentos, la FDA reportó un estimado de 2.4 a 0.16 µg/kg peso corporal/día para niños de 0 a 12 meses y de 0.19 µg/kg peso corporal/día para adultos [65].

1.5.1.2 Asociación con cáncer de mama

Por su condición de CDE, el BPA se ha asociado con un mayor riesgo de padecer cáncer de mama. Una de las primeras evidencias de que la exposición a CDEs estrogénicos podía incrementar el riesgo de cáncer de mama surgió a partir de la observación de que mujeres mayores de 40 años hijas de mujeres que habían recibido DES durante la gestación, presentaban una mayor incidencia de cáncer de mama en comparación con la población no expuesta [66].

Con respecto al BPA, múltiples estudios han encontrado que la exposición a este compuesto durante el periodo perinatal y prepuberal alteran el desarrollo de la glándula mamaria en roedores [67–69] e incluso en primates no humanos [70]. Es importante destacar que los efectos sobre el desarrollo de la glándula mamaria tienen repercusiones directas sobre la susceptibilidad al cáncer, al generar hiperplasia ductal, disminuir la apoptosis y generarse lesiones preneoplásicas en las glándulas mamarias [71,72].

1.5.1.3 Efectos sobre el sistema inmunológico

Con respecto a los efectos que el BPA ejerce sobre el sistema inmune, existe evidencia de que puede modificar la actividad de diversos tipos celulares, mas no se ha llegado a un consenso, probablemente porque los efectos observados dependen en gran medida de la dosis, el esquema de exposición y el contexto en el que se estudia. Sin embargo, se ha reportado que el BPA afecta a diversos tipos celulares del sistema inmunológico, modificando su porcentaje, funcionalidad, expresión de citocinas, moléculas de superficie y producción de inmunoglobulinas [73–78]. De forma interesante, existen reportes de que el BPA interfiere con la diferenciación de ciertos tipos celulares [79,80] e incluso con el desarrollo de órganos inmunes en aves [81].

2. Antecedentes

Como antecedente directo, un trabajo previo de nuestro grupo de investigación [82] encontró que la disrupción endócrina neonatal al cuarto día postnatal con una dosis farmacológica de 17β-estradiol resultó en cambios en la susceptibilidad a una infección parasitaria en la etapa adulta -revirtiendo incluso el dimorfismo sexual característico de dicha infección- y en la respuesta inmune asociada, incrementándose la expresión de IFN-γ en las hembras neonatalmente estrogenizadas e IL-4 en los machos neonatalmente estrogenizados.

Otro aspecto interesante fue el hallazgo de que esta disrupción modificó los patrones de expresión de los receptores de estrógenos y progesterona en el hipotálamo y el hipocampo.

Esto demostró que la disrupción endócrina neonatal es capaz de modificar permanentemente al sistema inmune, manifestándose dichos cambios ante un reto antigénico en la etapa adulta.

Uno de los principales mecanismos mediante los cuales los CDEs pueden causar efectos a largo plazo modificando la expresión genética es la alteración de los patrones epigenéticos. En este sentido, estudios recientes han confirmado que el BPA puede ocasionar cambios epigenéticos [83].

Particularmente, se ha reportado que la exposición a BPA durante periodos críticos del desarrollo puede modificar los patrones epigenéticos que controlan la expresión de receptores de estrógeno en tejidos reproductivos y en el cerebro [84–87].

Utilizando el modelo del pez cebra, el grupo de Santangeli y colaboradores [85] reportó que la exposición a BPA causó una regulación a la baja de la expresión de los genes que codifican para el receptor ERα en los ovarios, así como de otros genes involucrados en la esteroidogénesis, el crecimiento y maduración de los ovocitos.

Por otra parte, el grupo de Doshi [84] mostró que ratas macho expuestas a BPA durante los primeros cinco días posteriores al nacimiento presentaron una significativa hipermetilación de los promotores de los receptores ERα y ERβ, asociados a un incremento en la expresión de las metiltransferasas Dnmt3a y Dnmt3b.

En conjunto, existe evidencia de que la disrupción endócrina neonatal con un compuesto estrogénico puede modificar el comportamiento del sistema inmunológico ante un estímulo antigénico, a la vez que de manera particular el BPA puede modificar epigenéticamente la expresión de los receptores de estrógenos, los cuales son un elemento fundamental en la regulación a la que están sujetas las células del sistema inmunológico en el contexto de la red neuroinmunoendócrina.

3 Planteamiento del problema

Dado que una alteración endócrina durante el periodo crítico del desarrollo de un organismo puede modificar permanentemente la respuesta del sistema inmune a todo tipo de estímulos antigénicos, a que se ha documentado una constante exposición al BPA desde el período perinatal y a que se han encontrado asociaciones entre la exposición a BPA y el cáncer de mama, es de nuestro interés evaluar los efectos que la exposición neonatal de BPA ejerza sobre los principales componentes del sistema inmune involucrados en la inmunopatología tumoral en la etapa adulta.

4 Hipótesis

La exposición neonatal a Bisfenol A en el ratón modificará durante la etapa adulta la proporción de los linfocitos T reguladores, células NK, MATs y su función (secreción de citocinas IL-1β, IL-10, TGF-β, IFN-γ y TNF-α), lo que redundará en una mayor progresión de tumores mamarios inducidos experimentalmente.

5 Objetivos

5.1 Objetivo general

Identificar los efectos que se presenten en la respuesta inmune antitumoral en individuos que hayan sido expuestos neonatalmente al disruptor endócrino Bisfenol A.

5.2 Objetivos particulares

- Estudiar el efecto de la administración neonatal de BPA al tercer día postnatal (DPN) sobre el desarrollo de masas tumorales inducidas por la inoculación de células 4T1 en tejido mamario durante la etapa adulta, comparando la progresión y masa final de las tumoraciones producidas en los distintos grupos.
- Determinar por citometría de flujo los porcentajes de las principales subpoblaciones celulares involucradas en el control inmunológico del cáncer (NK, Treg, MATs) en bazo, nódulos linfáticos periféricos y masa tumoral en ratones controles y tratados neonatalmente con BPA.
- Evaluar la secreción de IL-10, IL-1β, TGF-β, IFN-γ y TNF-α en la masa tumoral en ratones controles y tratados neonatalmente con BPA.
- Analizar por citometría de flujo la expresión del receptor a estrógenos, isoforma alfa (ERα) en las células NK, Treg y MATs en bazo, nódulos linfáticos periféricos y masa tumoral en ratones controles y tratados neonatalmente con BPA.

6. Materiales y métodos

6.1 Ratones

En todos los experimentos se utilizaron ratones hembra de la cepa singénica BALB/c AnN (H-2^d) obtenidas de Harlan México (Facultad de Química, UNAM, México). Los animales se mantuvieron en las instalaciones de la Unidad de Modelos biológicos (UMB) del Instituto de Investigaciones Biomédicas (IIB) con condiciones controladas de temperatura (22°C) y ciclos luz-obscuridad de 12 horas, con agua y alimento Purina Diet 5015 (Purina, S. Louis, MO) *ad libitum*.

6.2 Declaración ética

El cuidado y las prácticas experimentales con los animales se llevaron a cabo en las instalaciones de la UMB del IIB, con la autorización de protocolo #155, en adherencia a las normas mexicanas (NOM-062-ZOO-1999) y con seguimiento a las recomendaciones hechas por el Instituto Nacional de Salud (NIH) de los Estados Unidos de América (Guide for the Care and Use of Laboratory Animals). La eutanasia de los animales experimentales se realizó de manera humanitaria, evitando al máximo el sufrimiento.

6.3 Administración neonatal de BPA

Se determinó la fecha de nacimiento, designando al día de hallazgo de camada como día 0.5 postnatal. Al tercer día postnatal se identificó a las crías hembra mediante medición de distancia ano-genital y cada camada se asignó a uno de tres grupos experimentales. El grupo *Intacto* no recibió ningún tratamiento neonatal. Las crías hembras del grupo *Vehículo* recibieron una inyección subcutánea de 20 µl de aceite de maíz (Sigma, St. Louis MO). Las crías hembras del grupo *BPA* recibieron una inyección de BPA equivalente a 250 µg/kg de peso corporal. En cada experimento independiente se asignaron entre 8 y 10 crías por cada grupo.

6.4 Evaluación de parámetros endócrinos

6.4.1 Apertura vaginal

Este parámetro se registró como indicativo del inicio de la pubertad de los ratones hembra. A partir de los 28 días de nacidas, todas las hembras de los tres grupos fueron inspeccionadas en búsqueda de apertura vaginal mediante sujeción dorsal y ligera extensión con los dedos índice y pulgar de la piel alrededor del área vaginal. Se registró la fecha en la que se observó la apertura de la cavidad vaginal.

6.4.2 Ciclo estral

Una vez establecida plenamente la madurez sexual, a partir de las 8 semanas de edad, se seleccionaron 4 hembras de cada grupo experimental para evaluar su ciclo estral mediante un lavado vaginal con 50 µl de solución salina al 0.9% (PiSA, Guadalajara México), con el cual se realizó un frotis en portaobjetos que posteriormente fue teñido con Giemsa y observado en el microscopio.

Se asignó la fase del ciclo estral correspondiente a cada frotis con base en los siguientes criterios de interpretación:

- Proestro: predominancia de células epiteliales nucleadas ocasionalmente acompañadas de células epiteliales cornificadas. Leucocitos escasos o ausentes.
- Estro: abundantes células epiteliales cornificadas escamosas, en cúmulos.
- Metaestro: predominancia de leucocitos con presencia de células epiteliales cornificadas escamosas y ocasional presencia de escasas células epiteliales nucleadas.
- Diestro: abundancia de leucocitos con ocasional presencia de escasas células epiteliales nucleadas.

6.4.3 Niveles séricos de 17β-estradiol

Se utilizaron muestras de suero obtenidas durante el sacrificio, correspondientes a la fase de diestro y se determinaron los niveles séricos de estradiol utilizando el kit de EIA DetectX® Serum 17β-Estradiol (Arbor Assays, Ann Arbor MI) de acuerdo a las instrucciones del fabricante.

6.5 Modelo de adenocarcinoma mamario murino: línea 4T1

6.5.1 Cultivo

La línea celular 4T1 (ATCC® CRL-2539) fue amablemente donada por el Dr. Pedro Ostoa-Saloma y cultivada en medio RPMI 1640 (Sigma, St. Louis MO) con 10% de Suero Fetal Bovino (By Productos, Guadalajara, México). Al alcanzar el 70-80% de confluencia, se procedía al subcultivo mediante la retirada del medio, dos lavados con PBS y la incubación con una solución de Tripsina-EDTA 0.25% (Gibco, Grand Island NY) durante 5 minutos.

Después del segundo subcultivo, las células fueron cosechadas, suspendidas en solución salina al 0.9% (PiSA, Guadalajara México), contadas y llevadas a una concentración de 250,000 células/ml para ser inoculadas en los ratones.

6.5.2 Inoculación

Los ratones fueron sometidos a anestesia mediante la inhalación de una mezcla de aire y sevofluorano (Abbot, México DF) al 5%, tras lo cual se procedió a la asepsia del vientre bajo, se localizó el 4° pezón y con una jeringa de 1 ml con aguja 27G se procedió a la inyección de 40 µl de suspensión celular, equivalente a 10,000 células, en el cojinete graso de la glándula mamaria inguinal. Se monitoreó el desarrollo de la masa tumoral durante 25 días, tras los cuales se procedió a la eutanasia.

En cada experimento independiente se inoculó a 4 ratones de cada uno de los grupos experimentales definidos por la intervención neonatal (*Intacto*, *Vehículo* y *BPA*), resultando en los siguientes grupos:

- *Intacto-control*
- *Intacto-4T1*
- *Vehículo-control*
- *Vehículo-4T1*
- *BPA-control*
- *BPA-4T1*

6.6 Obtención de muestras

Transcurridos los 25 días de desarrollo tumoral, los ratones se sacrificaron para la obtención y procesamiento de los tejidos mencionados a continuación. El sacrificio se condujo de manera humanitaria, sometiendo a los ratones a anestesia con sevofluorano, procediendo posteriormente a la punción cardíaca que condujo a la muerte por hipovolemia.

6.6.1 Suero

Este procedimiento se realizó estando cada animal bajo anestesia, utilizando una jeringa de 1 ml con aguja desprendible de calibre 27G. La aguja se retiró para colocar la sangre recolectada en tubos de microcentrífuga de 1.5 ml, se permitió que ocurriera la coagulación de la misma y posteriormente se centrifugó a 3,500 rpm, a 4°C, durante 10 minutos. Una vez separado el suero, se hicieron alícuotas de 20 µl y se almacenaron a -20°C hasta su análisis.

6.6.2 Órganos linfoides

Posterior a la punción cardíaca, se procedió a la disección de los órganos linfoides: bazo y nódulos linfáticos inguinales. Éstos se colocaron en pozos de placa de cultivo con 1 ml de PBS y se mantuvieron sobre hielo.

Los nódulos y la mitad del bazo se mantuvieron en frío en PBS hasta su procesamiento para citometría de flujo y la otra porción de bazo se colocó en un tubo de microcentrífuga con 500 µl de reactivo TRIzol (Ambion, Carlsbad CA), tras lo cual se colocó en hielo seco para congelarlo rápidamente. Posteriormente, las muestras de bazo congeladas se almacenaron a -70°C hasta su posterior procesamiento para extracción de RNA.

6.6.3 Tumor

Para la disección del tumor, se realizó una incisión sobre la línea media abdominal y dos más desde el extremo caudal de la incisión hacia las patas traseras; con ayuda de pinzas y tijeras de punta redonda se realizó una disección roma para exponer la cara interna de la piel, en donde se ubica el tumor. Utilizando un bisturí, se procedió a separar el tumor de la piel, cuidando de cortar únicamente el tejido conectivo que los une.

Una vez separado el tumor de la piel, se pesó en una balanza analítica (Kern & Sohn, GmBH) y se registró el peso. Posteriormente se dividió en tres partes iguales; una de ellas se almacenó en un tubo de microcentrífuga con reactivo TRIzol y se congeló en hielo seco inmediatamente, otra parte se mantuvo en frío en PBS hasta su procesamiento para citometría de flujo y la tercera porción se procesó para inmunofluorescencia.

6.7 Citometría de flujo

El bazo y los nódulos linfáticos periféricos inguinales se disgregaron mediante el paso por una malla de nylon. Los eritrocitos del bazo se lisaron incubando 10 minutos en buffer de lisis de eritrocitos (0.15 M NH_4Cl, 1 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3). Las muestras de tejido tumoral se picaron finamente y se incubaron durante 20 minutos en medio de digestión (RPMI 1640, 10 U/ml DNasa I (Roche, Mannheim Germany), 0.5 mg/ml Colagenasa IV (Sigma, St. Louis MO)), la digestión se detuvo añadiendo 50 µl de SFB y se procedió a la disgregación con malla. Las células de todos los tejidos se lavaron

y suspendieron en buffer de tinción (solución amortiguadora de fosfatos (PBS), 2% SFB, 0.02% NaN_3).

Las tinciones se realizaron en placas de 96 pozos con fondo U. En cada pozo se colocó una cantidad de suspensión celular equivalente a 1 x 10^6 células y se incubaron durante 20 minutos a 4°C con anticuerpo anti-CD16/CD32 (TruStain®, BioLegend, San Diego CA) para bloquear los receptores FC (CD16 y CD32) de la superficie celular y evitar una tinción inespecífica. Posteriormente, se realizó un lavado con buffer de tinción y se incubaron durante 20 minutos a 4°C con los anticuerpos para tinción de superficie (ver **Tabla 1**), protegidas de la luz. Una vez teñida la superficie celular, las células se lavaron con buffer de tinción, se fijaron y permeabilizaron con el kit de fijación y permeabilización para FOXP3 (Tonbo biosciences, San Diego CA) de acuerdo a las instrucciones del fabricante. Una vez permeabilizadas, las células se incubaron durante 30 minutos a 4°C con los anticuerpos para tinción intracelular (ver **Tabla 1**), protegidas de la luz. Posteriormente, se lavaron con buffer de permeabilización y se incubaron durante 20 minutos a 4°C con el anticuerpo secundario (ver **Tabla 1**). Finalmente, las células se lavaron con buffer de permeabilización y se resuspendieron en 200 µl de buffer de tinción. Las células teñidas se capturaron utilizando el citómetro Attune (Life Technologies) y se analizaron con el software FlowJo® (Tree Star Inc.).

La identificación de las subpoblaciones celulares se llevó a cabo mediante las estrategias de selección de regiones ilustradas en las **figuras 2-5**.

Tabla 1. Anticuerpos utilizados en las tinciones para citometría de flujo.

Anticuerpo	Clona	Fluoróforo	Marca
Anti-CD3	145-2C11	APC Cy®7	Biolegend, San Diego CA
Anti-CD4	GK1.5	PE	Biolegend, San Diego CA
Anti-CD8	53-6.7	PerCP	Biolegend, San Diego CA
Anti-CD25	PC61.5	VioletFluor®450	Tonbo biosciences, San Digeo CA
Anti-Foxp3	150D	AlexaFluor®647	Biolegend, San Diego CA
Anti-CD19	6D5	PE	Biolegend, San Diego CA
Anti-F4/80	BM8	AlexaFluor®647	Biolegend, San Diego CA
Anti-NKp46	29A1.4	PE	Biolegend, San Diego CA
Anti-ERα	H-184	-	Santa Cruz bt., Dallas TX
Donkey anti-Rabbit IgG	Poly4064	DyLight®488	Biolegend, San Diego CA

APC Cy7, aloficocianina acoplada a Cychrome® 7; PE, ficoeritrina; PerCP, proteína clorofila peridinina

Para las subpoblaciones de linfocitos, a partir de las características de tamaño y complejidad asociadas a los parámetros FSC-A y SSC-A, respectivamente, se seleccionó el área correspondiente a estas células, como se muestra en el panel superior izquierdo de la **figura 2**. Posteriormente, dentro de esta región se modificó el parámetro de las ordenadas de modo que mostrara el canal de fluorescencia correspondiente al fluoróforo APC Cy7, con el que se tiñó la molécula CD3 y se seleccionó la población positiva a este marcador, característico de los linfocitos T (**fig. 2,** panel superior derecho). Dentro de esta región, observando los parámetros de fluorescencia correspondientes a PE y PerCP, con los que se tiñeron los correceptores CD4 y CD8, respectivamente, se seleccionaron las poblaciones positivas a estos marcadores, determinantes de los linfocitos T cooperadores y los linfocitos T citotóxicos, respectivamente (**fig. 2**, panel inferior derecho). Finalmente, para identificar a los linfocitos T reguladores, se utilizaron dos marcadores: CD25 y Foxp3 cuya cotinción, dentro de la región $CD4^+$, determinó la identidad de los Treg (**fig. 2**, panel inferior izquierdo).

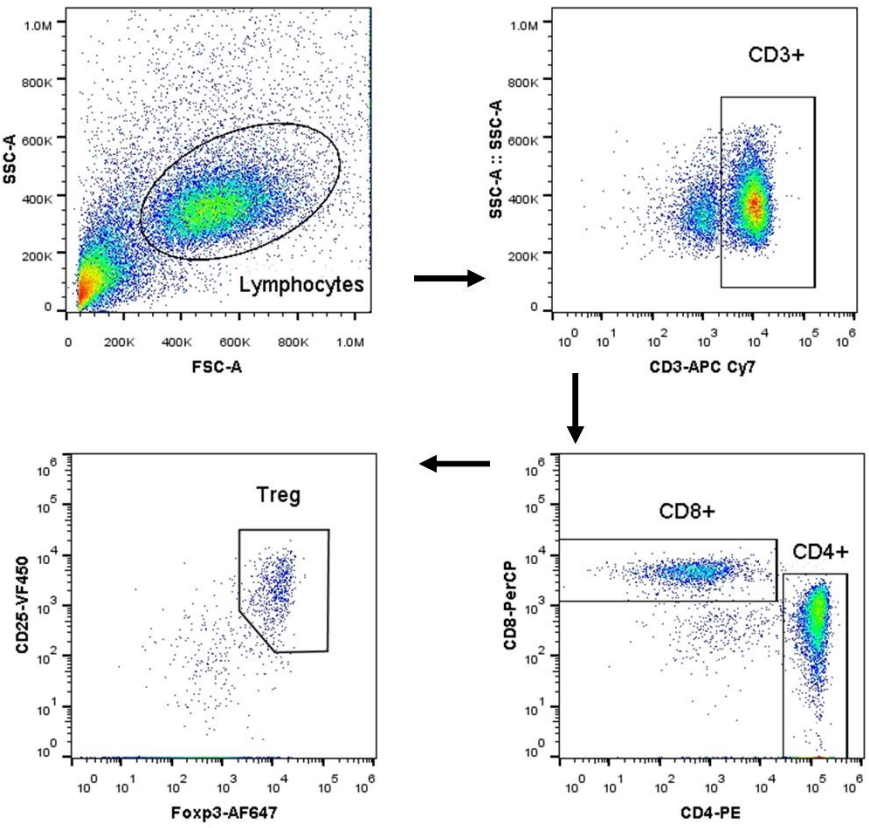

Figura 2. Estrategia de identificación de subpoblaciones de linfocitos T. *Dot plots* representativos de la secuencia de selección de regiones de interés. Datos analizados con el software Flow Jo.

La identificación de los linfocitos B consistió únicamente en la ubicación de la población $CD19^{+}$ dentro de la región correspondiente a los linfocitos, como se muestra en la **figura 3**.

Como se muestra en la **figura 4**, para la identificación de las células NK se seleccionó un área similar a la asociada a los linfocitos, pero abarcando células más

grandes y complejas. Posteriormente se seleccionó la población NKp46+ dentro de la misma.

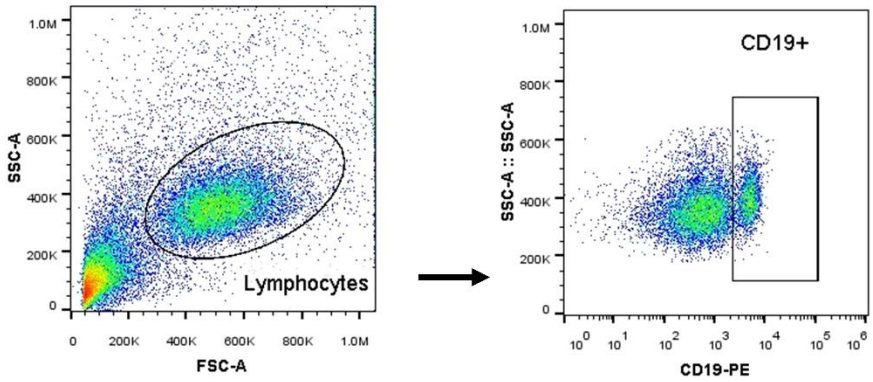

Figura 3. Estrategia de identificación de linfocitos B. *Dot plots* representativos de las regiones de interés. Datos analizados con el software Flow Jo.

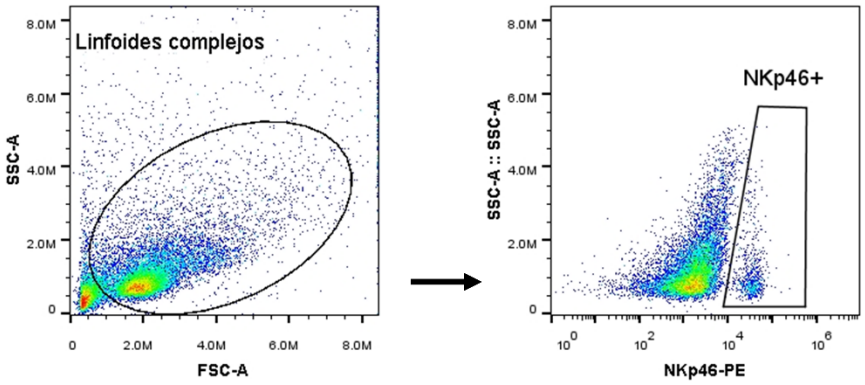

Figura 4. Estrategia de identificación de células NK. *Dot plots* representativos de las regiones de interés. Datos analizados con el software Flow Jo.

Para la selección de los macrófagos, con base en los parámetros de tamaño y complejidad se seleccionó una región correspondiente a las células de mayor tamaño y complejidad, excluyendo a las células linfoides de menor tamaño. Dentro de esta región se localizaron las células positivas al marcador F4/80, como se aprecia en la **figura 5**.

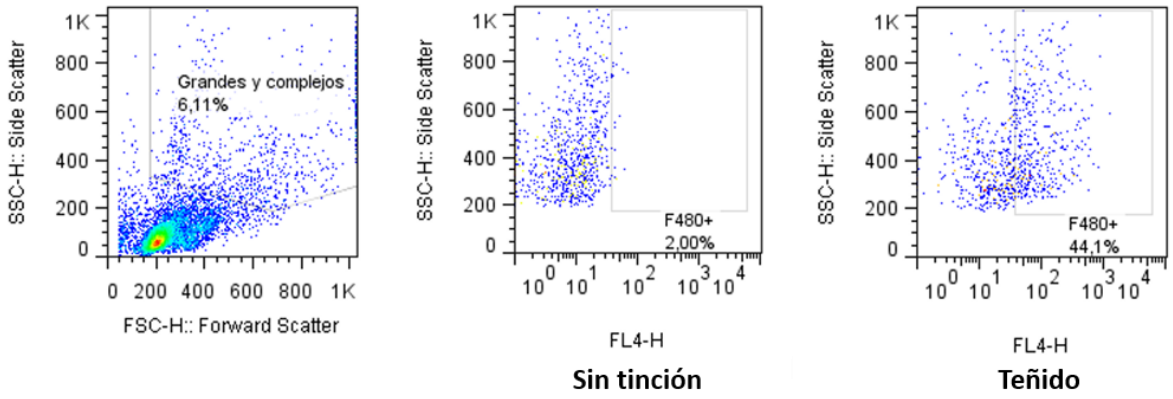

Figura 5. Estrategia de identificación de macrófagos. *Dot plots* representativos de las regiones de interés. Datos analizados con el software Flow Jo.

Finalmente, dentro de cada población se determinó el porcentaje de células positivas al receptor ERα, así como la intensidad mediana de fluorescencia de las células positivas.

6.8 Inmunofluorescencia

Las muestras de tejido tumoral se fijaron en paraformaldehído al 4% en PBS (pH 7.1) durante 20 minutos, se lavaron con PBS y se sometieron a crioprotección sumergiéndolos en sacarosa al 30% en PBS durante toda la noche en refrigeración, se embebieron en compuesto O.C.T. TissueTek (Sakura Finetek, Torrance CA) y se congelaron a -70°C.

Se realizaron cortes de 10 µm en criostato, las cuales se montaron en portaobjetos cargados. Los portaobjetos con los cortes se colocaron en una cámara de vacío durante una hora para asegurar la adherencia de los cortes. Los cortes se hidrataron cubriéndolos con PBS y retirando el mismo mediante el uso de un dispositivo de vacío. Posteriormente, se sometieron a un proceso de permeabilización cubriéndolos con Triton X-100 (Sigma, St. Louis MO) al 1% en PBS durante 10 minutos. Transcurrido este tiempo

se retiró el Triton X-100 y se lavaron dos veces cubriéndolos con PBS, esperando 5 minutos y retirando el mismo. Los cortes se bloquearon cubriéndolos con solución de albúmina de suero bovino (Sigma, St. Louis MO) al 1% en PBS durante 2 horas. Después de retirar la solución de albúmina, los cortes se incubaron durante 1 hora a 4°C, protegidos de la luz, con los anticuerpos anti-CD4-PE (GK1.5) [1:300] y anti-Foxp3-AlexaFluor®647 (150D) [1:100] (BioLegend, San Diego CA) diluidos en solución de albúmina. Posterior a ello, los cortes se lavaron 4 veces con PBS y se montaron con medio histológico Fluoroshield (Sigma, St. Louis MO). Los cortes se almacenaron a 4°C, protegidos de la luz, hasta su observación en el microscopio confocal LSM5 Pascal (Carl Zeiss).

6.9 RT-PCR

6.9.1 Extracción de RNA

La obtención de RNA de las muestras de bazo y tumor se realizó por la técnica de extracción orgánica con reactivo TRIzol siguiendo las instrucciones del fabricante.

Las muestras de tejido almacenadas en TRIzol a -70°C se descongelaron lentamente en hielo y se cortaron en trozos lo más pequeños posible con ayuda de pinzas y bisturí, tras lo cual se colocaron en tubos de ensaye de vidrio estériles, agregando 1 ml de TRIzol por cada 100 mg de tejido y se mantuvieron en hielo hasta su homogenización. Las cuchillas del homogenizador se limpiaron con una serie de soluciones: saturada de NaCl, etanol al 75%, agua libre de RNasas (tratada con DEPC) y TRIzol. Una vez limpias las cuchillas, se introdujeron en el tubo de ensaye y se procedió a la homogenización de las muestras de tejido. Entre cada muestra se limpió el homogenizador con agua libre de RNasas y TRIzol. El homogenizado se transfirió a tubos de microcentrífuga de 1.5 ml y se agregaron 200 µl de fenol-cloroformo 5:1 (Amresco) por cada ml de homogenizado. La mezcla se agitó en vórtex durante 30 segundos, tras lo cual se centrifugó a 13,000 rpm a 4°C, durante 15 minutos. Se recuperó la fase acuosa en un nuevo tubo de microcentrífuga, se añadieron 200 µl de cloroformo (Sigma) y se agitó en vórtex durante 30 segundos, tras lo cual se centrifugó a 13,000 rpm a 4°C, durante 15 minutos. Se

recuperó la fase acuosa y en un nuevo tubo de microcentrífuga y se le agregó un volumen igual de isopropanol (Sigma) a 4°C. Esta mezcla se mantuvo a 4°C durante un mínimo de 8 horas para permitir la precipitación de RNA. Posteriormente se centrifugó a 13,000 rpm a 4°C durante 15 minutos, se decantó el isopropanol y se lavó la pastilla de RNA con etanol al 75% en agua libre de RNasas. Se centrifugó nuevamente bajo las mismas condiciones, durante 5 minutos, se retiró el etanol con ayuda de una micropipeta y se colocó cada tubo sobre una gasa estéril para permitir la evaporación de los residuos de etanol. La pastilla de RNA se disolvió agregando entre 20 y 50 µl de agua libre de RNasas de acuerdo a su tamaño.

Para determinar la concentración del RNA obtenido, se hizo una dilución 1:70 de cada muestra de RNA en agua libre de RNasas y se leyó en un espectrofotómetro (GeneQuant) a 260 y 280 nm por duplicado. Mediante la fórmula general (RNA = ABS_{260} * D.O. * dilución = ng/ µl) utilizando el software del espectrofotómetro, se obtuvo la concentración de cada muestra.

La integridad del RNA extraído se verificó corriendo 2 µg de RNA en un gel de agarosa al 1.5% + 5 µl de SYBR Safe (Thermo Fisher Scientific) por cada 100 µl de gel.

6.9.2 Retrotranscripción

El RNA obtenido se retrotranscribió a cDNA utilizando el kit de M-MLV Reverse Transcriptase (Invitrogen, Carlsbad CA) de acuerdo a las instrucciones del fabricante.

Se hicieron dos mezclas de reactivos, escalando las cantidades por cada reacción (nX), como se muestra en la **Tabla 2**.

Por cada muestra se agregó a un microtubo de PCR lo siguiente: 2.5 µl de Mix A, 5 µg de RNA y agua libre de RNasas, c.b.p. 17 µl. Los tubos se introdujeron al termociclador GenAmp (Applied biosystems) y se mantuvieron a 65°C durante 5 minutos, posteriormente se retiraron del termociclador y se mantuvieron el hielo durante 3 minutos, tras los cuales se agregaron 8 µl de Mix B a cada uno. Una vez que el termociclador se encontró a 37°C, se introdujeron los tubos y se mantuvieron a esa temperatura durante

50 minutos. Una vez terminada la reacción, el cDNA obtenido se almacenó a -20°C hasta su uso para amplificación por PCR.

Tabla 2. Mezclas de reactivos para la reacción de retrotranscripción de RNA.

Reactivo	**1X**
Mix A	
Oligo $(dT)_{12-18}$ 0.5 µg/µl	0.5 µl
dNTPs 10 mM c/u	2 µl
Mix B	
Buffer FS 5X	5 µl
DTT 0.1 M	2 µl
M-MLV RT 200 U/ µl	1 µl

6.9.3 PCR

Una vez obtenido el cDNA, se procedió a la amplificación de los genes de interés mediante la reacción de cadena de la polimerasa (PCR) utilizando el kit de Taq DNA Polimerasa (Biotecnologías universitarias, México), siguiendo instrucciones del fabricante. Brevemente, por cada reacción se preparó una mezcla de reactivos, escalando las cantidades por cada reacción (nX), como se muestra en la **Tabla 3**.

Tabla 3. Mezclas de reactivos para la reacción de PCR.

Reactivo	**1X**
Amortiguador 10X	2.5 µl
$MgCl_2$ 30 mM	1.25 µl
dNTPs 10 mM c/u	2 µl
Oligo Sentido 20 µM	1.7 µl
Oligo Antisentido 20 µM	1.7 µl
H2O libre de RNasas	13.6 µl
Taq DNA pol. 5 U/µl	0.25 µl

Se agregaron 23 µl de mezcla de reacción a cada microtubo para PCR y se añadieron 2 µl de cDNA. Posteriormente se introdujeron al termociclador GenAmp y se procesaron de acuerdo al siguiente programa general:

95°C	5 min	
94°C	1 min	n ciclos
TM	1 min	
72°C	1 min	
72°C	7 min	
4°C	∞	

Las secuencias de oligonucleótidos utilizados, así como la TM, ciclos y tamaño del producto obtenido se detallan en la **Tabla 4**.

Una vez obtenidos los amplificados, se corrieron en un gel de agarosa al 1.5% + 5 µl de SYBR Safe (Thermo Fisher Scientific) por cada 100 µl de gel. Para normalizar el nivel de expresión con respecto al gen de expresión constitutiva (18S), los amplificados de cada gen de interés se corrieron paralelamente con los amplificados de la subunidad 18S. Una vez terminado el corrimiento electroforético, se capturó cada gel con el fotodocumentador MultiDoc-It (UVP, Upland CA) y se obtuvieron los valores de densidad óptica de cada banda utilizando el software ImageJ (National Institutes of Health, USA). La expresión normalizada de cada gen se obtuvo mediante el cociente de la densidad óptica de la banda del gen de interés entre la intensidad de la banda de 18S de cada muestra.

Tabla 4. Oligonucleótidos utilizados para las reacciones de PCR.

Oligonucleótido	Secuencia	TM (°C)	Ciclos	Producto (pb)
IL-1β Sentido	TCATGGGATGATGATGATAACCTGCT	62	35	502
IL-1β Antisentido	CCCATACTTTAGGAAGACACGGATT			
IL-10 Sentido	AACTGGTAGAAGTGATGCCCCAGGCA	63	35	237
IL-10 Antisentido	CTATGCAGTTGATGAAGATGTCAAA			
TNF-α Sentido	GGCAGGTCTACTTTGGAGTCATTGC	63	30	300
TNF-α Antisentido	ACATTCGAGGCTCCAGTGAATTCGG			
IFN-γ Sentido	AGCGGCTGACTGAACTCAGATTGTAG	60	30	247
IFN-γ Antisentido	GTCACAGTTTTCAGCTGTATAGGG			
TGF-β Sentido	CTTCAGCTCCACAGAGAAGAACTGA	61	30	298
TGF-β Antisentido	CACAATCATGTTGGACAACTGCTCC			
18S Sentido	CGCGGTTCTATTTTGTTGGT	60	25	219
18S Antisentido	AGTCGGCATCGTTTATGGTC			
Arg-1 Sentido	CTTGCGAGACGTAGACCCTG	64	40	387
Arg-1 Antisentido	TGAGTTCCGAAGCAAGCCAA			
Fizz-1 Sentido	GGTCCCAGTGCATATGGATGAGAC	58	40	296
Fizz-1 Antisentido	CACCTCTTCACTCGAGGGACAGTT			
YM-1 Sentido	TCACAGGTCTGGCAATTCTTCTG	60	40	437
YM-1 Antisentido	TTTGTCCTTAGGAGGGCTTCCTC			
iNOS Sentido	CAGCTCCACAAGCTGGCTCG	63	40	657
iNOS Antisentido	CAGGATGTCCTGAACGTAGACCTT			

7. Resultados

7.1 Evaluación de parámetros endócrinos

Dado que la estrategia experimental involucra el desarrollo de un tumor en un tejido sensible a hormonas, era importante determinar si los efectos observados se debían a una alteración hormonal persistente o al efecto directo o indirecto de la disrupción endócrina neonatal sobre el sistema inmunológico.

Para evaluar los potenciales efectos reproductivos de una única administración neonatal de 250 µg/kg de peso corporal de BPA, se monitoreó el inicio de la pubertad, determinada por la edad a la cual ocurre la apertura vaginal. Como se muestra en la **figura 6**, la exposición neonatal a esta dosis de BPA no alteró el inicio de la pubertad, como se ha reportado con dosis más altas o con exposiciones prolongadas.

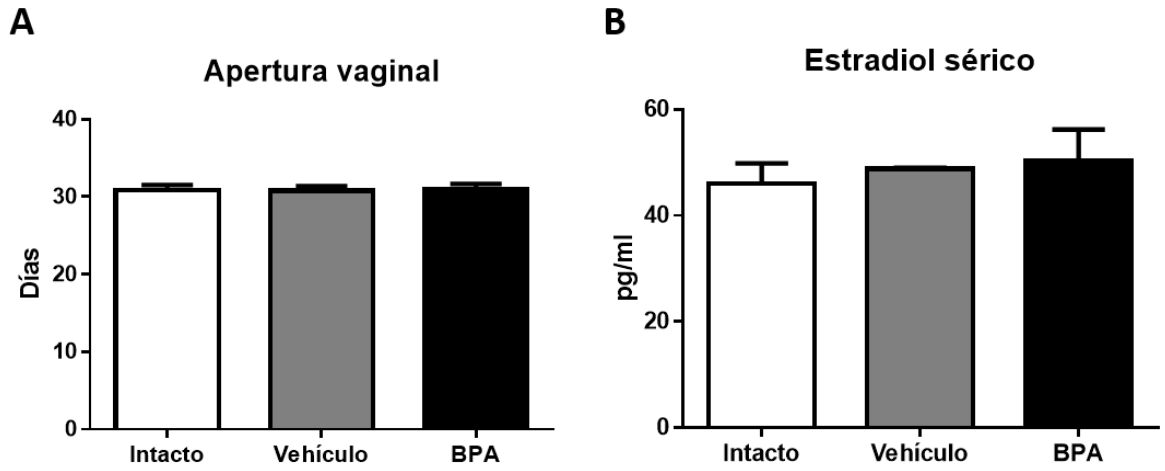

Figura 6. Evaluación de parámetros endócrinos. **A**: edad de presentación de la apertura vaginal como indicador del inicio de la pubertad; **B:** niveles séricos de 17β-Estradiol en fase de diestro a las 12 semanas de edad, determinados por EIA. Datos de 2 experimentos independientes expresados como media ± DE; n = 14 por grupo en **A** y 8 por grupo en **B**.

Una vez que los ratones alcanzaron la plena madurez sexual (8 semanas de edad) se realizó un seguimiento del ciclo estral. Si bien se observaron algunos individuos con ciclos no canónicos (caracterizados por un diestro prolongado de 3 o 4 días con evolución a estro), estos se encontraban distribuidos entre los 3 grupos experimentales de exposición (*Intacto*, *Vehículo* y *BPA*) y no representaban más de un 16% de cada grupo, por lo que se determinó que no existieron diferencias entre los grupos experimentales.

Adicionalmente, la exposición a BPA no tuvo efecto sobre los niveles basales de estradiol sérico, determinados en la fase de diestro, como se observa en la **figura 6**.

7.2 Evaluación de tumores mamarios

A partir de los 7 días posteriores a la inoculación de 10,000 células de la línea 4T1, se monitoreó el desarrollo tumoral registrando el diámetro mayor de la masa tumoral palpable a través de la piel. En la **figura 7 A** se muestra el seguimiento del tamaño superficial de las masas tumorales hasta el día 20 post-inoculación, el cual no muestra diferencias entre los tres grupos.

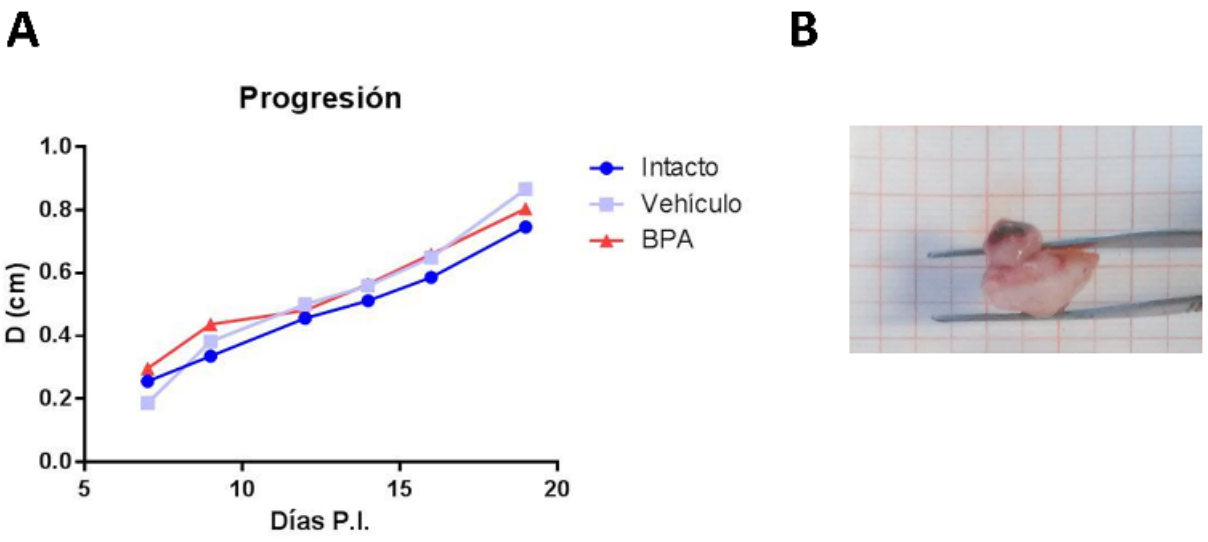

Figura 7. Seguimiento del desarrollo tumoral. Una vez evidente el surgimiento de los tumores, éstos fueron medidos con vernier. **A**: Diámetro mayor (D) en centímetros (cm) de las masas tumorales; **B**: imagen representativa de la disposición irregular de las masas tumorales que no permitió correlacionar el seguimiento temporal con el desarrollo final de los tumores.

Sin embargo, esta medida se descartó para el resto de los experimentos, pues no refleja el tamaño total de la masa tumoral, como se puede observar en la **figura 7 B**.

Transcurridos 25 días de desarrollo tumoral, al momento del sacrificio, se compararon las masas tumorales. La **figura 8** muestra las imágenes representativas de los tumores correspondientes a los tres grupos experimentales y permite observar que el grupo expuesto a BPA presentaba tumores de mayor tamaño. Dada la irregularidad de las masas tumorales, la magnitud del desarrollo tumoral se determinó mediante el peso de las mismas, el cual se normalizó con respecto al control para homogenizar los resultados entre experimentos independientes. Como se muestra en la **figura 8,** los tumores del grupo BPA se desarrollaron hasta obtener un peso 88% mayor que en el grupo intacto. La diferencia fue significativa contra los dos grupos no expuestos a BPA, con una media de 1 (I.C. = 0.8 – 1.19) para el grupo Intacto, 1.12 (I.C. = 0.98 – 1.25) para el grupo Vehículo y 1.88 (I.C. = 1.48 – 2.28) para el grupo BPA.

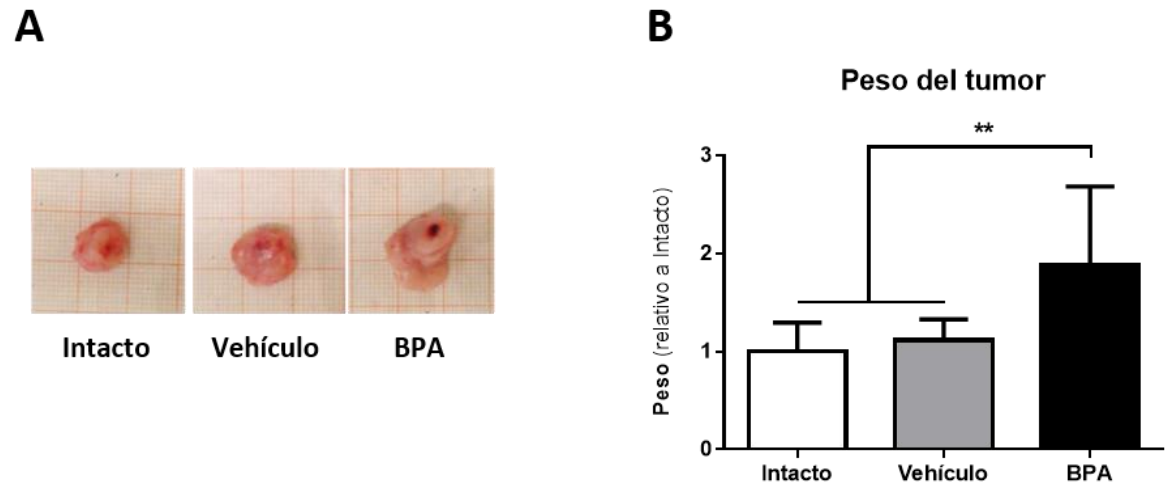

Figura 8. Crecimiento tumoral. **A**: imágenes representativas de los tumores en los tres grupos experimentales, cuadrícula milimétrica de fondo; **B**: evaluación del peso final de los tumores tras 25 días de desarrollo. Datos de 3 experimentos independientes expresados como media ± DE; n = 12 por grupo; ** p = 0.007 vs Intacto, p = 0.0025 vs Vehículo.

7.3 Análisis de subpoblaciones celulares

Se analizaron distintas subpoblaciones celulares a nivel local, regional y sistémico mediante tinciones de citometría del infiltrado leucocitario tumoral, nódulos linfáticos periféricos drenantes y bazo de los sujetos experimentales.

7.3.1 Infiltrado tumoral

La **figura 9** muestra las subpoblaciones analizadas en tejido tumoral. Como se puede apreciar, la composición celular es homogénea entre los grupos (**fig. 9 A-C**), con excepción de los linfocitos T reguladores, los cuales se encontraban incrementados en el grupo expuesto a BPA (**fig. 9 D-E**), constituyendo un 13.93% (I.C. 10.06, 17.79) de los linfocitos $CD3^+$ $CD4^+$, en comparación con un 7.82% (I.C. 5.77, 9.87) y 7.33% (I.C. 3.81, 10.85) en los grupos Intacto y Vehículo, respectivamente.

Sin embargo, incluso tras la digestión y disgregación de las muestras de tejido tumoral, la cantidad de células del infiltrado leucocitario era baja y se planteó la posibilidad de que no representaran adecuadamente al mismo. Por ello se realizó un análisis adicional por inmunofluorescencia para la identificación de los linfocitos T reguladores, la cual parecía ser una subpoblación de interés.

Como se puede observar en la **figura 10**, la tinción por inmunofluorescencia confirmó que en el grupo expuesto a BPA existe una mayor proporción de linfocitos T reguladores, representando un promedio de 42.8% (I.C. 30.27, 55.33) de los linfocitos T $CD4^+$, en comparación con un 21.54% (I.C. 14.14, 28.98) en el grupo Intacto y 21.88% (I.C. 11.52, 30.64) en el grupo Vehículo.

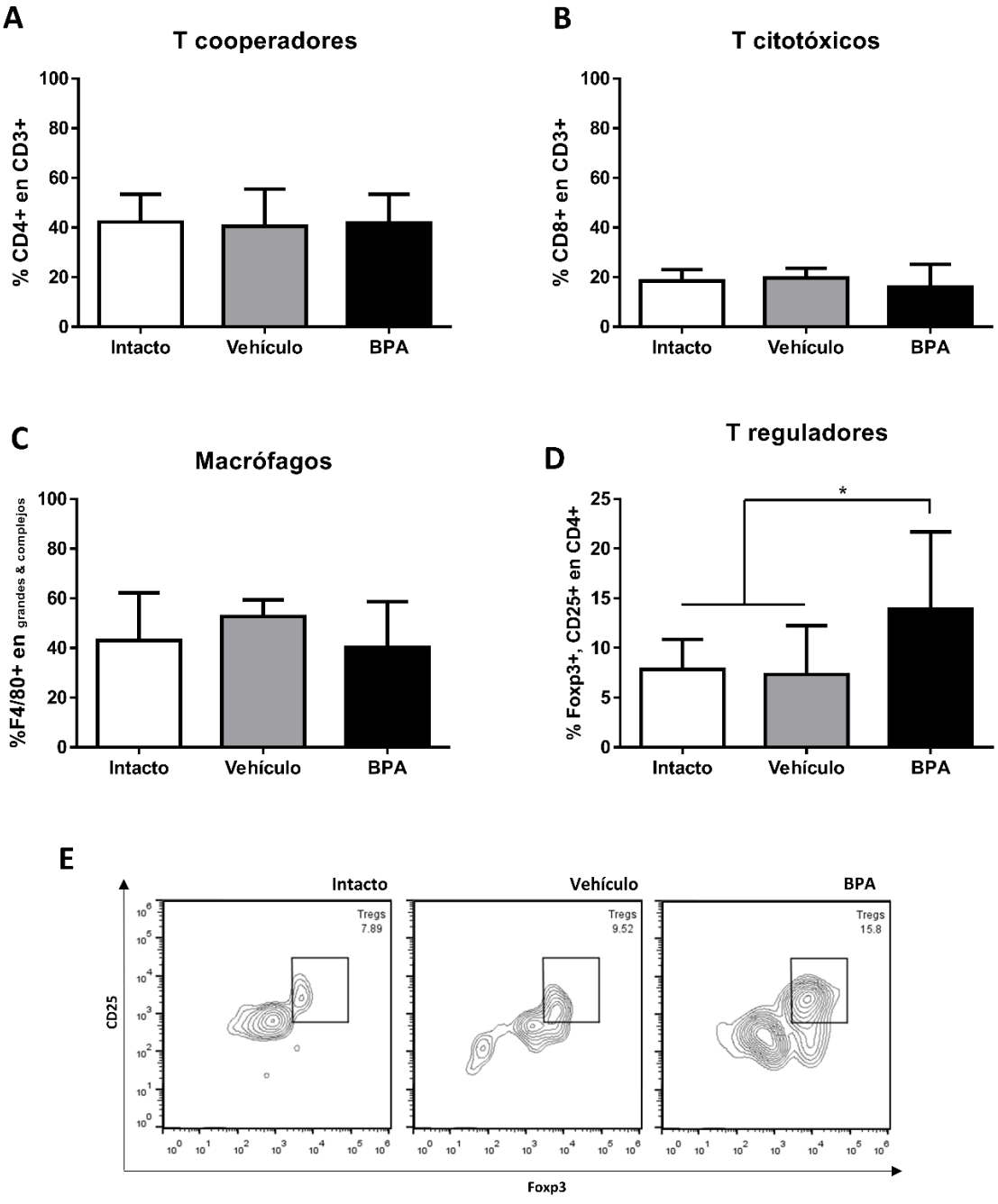

Figura 9. Análisis del infiltrado leucocitario tumoral. Determinación de subpoblaciones inmunológicas por citometría de flujo. **A**: linfocitos T cooperadores; **B**: linfocitos T citotóxicos; **C**: macrófagos; **D**: linfocitos T reguladores, * p = 0.0335 vs Intacto, p = 0.0247 vs Vehículo. Datos de 3 experimentos independientes expresados como media ± DE; n = 12 por grupo; **E**: capturas representativas del análisis del porcentaje de Treg, región: $CD3^+$, $CD4^+$ provenientes de 10,000 linfocitos colectados.

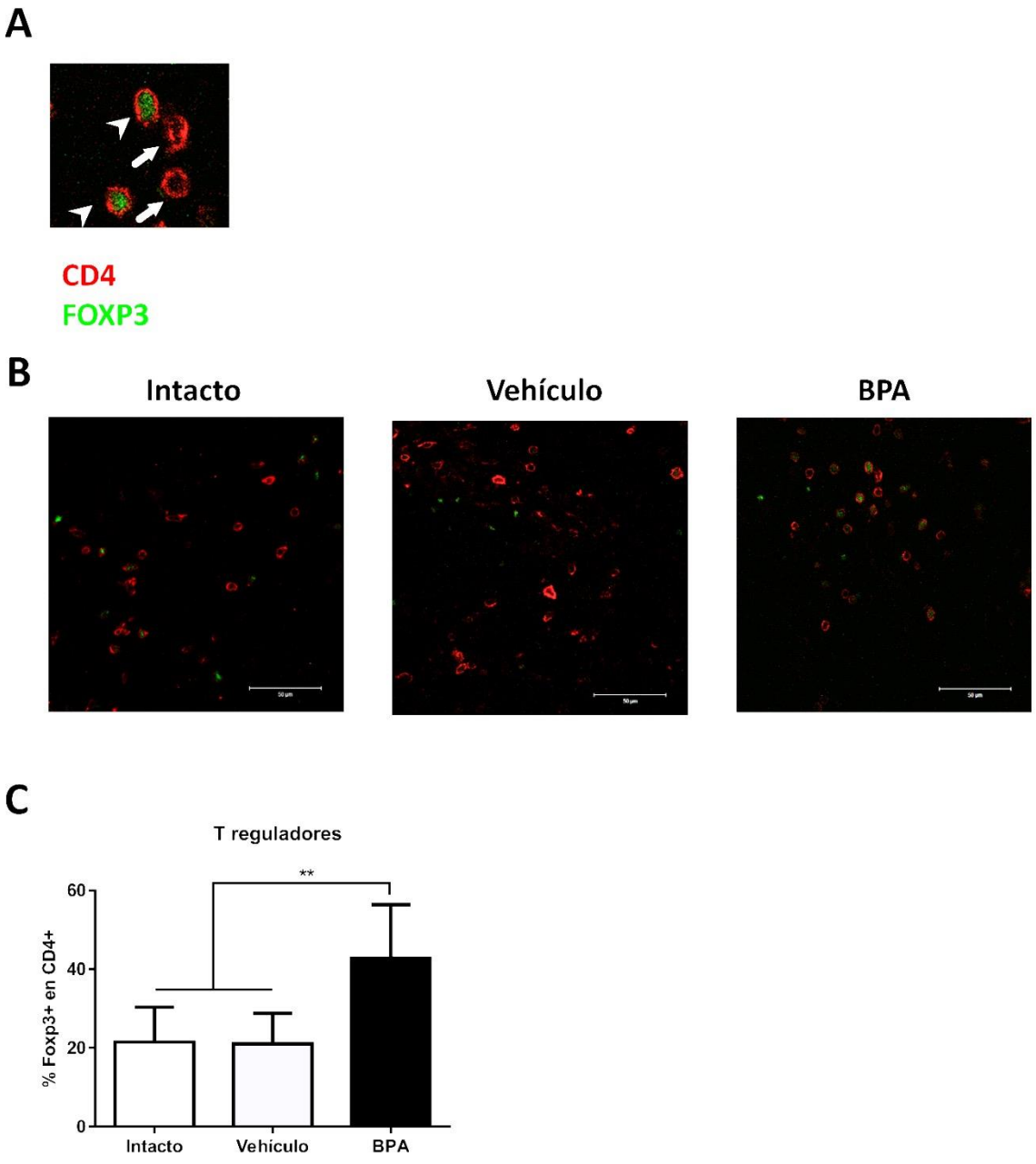

Figura 10. Evaluación de la abundancia de linfocitos T reguladores por inmunofluorescencia. **A**: identificación representativa de T helper (flechas) y Treg (puntas de flecha) mediante tinción membranal de CD4 (rojo) y nuclear de Foxp3 (verde); **B**: imágenes representativas de la tinción en las muestras de tumores, barra de escala: 50 µm, aumento original: 40X; **C**: porcentaje de Treg expresado como el porcentaje de células $Foxp3^+$ en las células $CD4^+$. Datos de 2 experimentos independientes expresados como media ± DE; n = 8 por grupo; ** p = 0.0031 vs Intacto, p = 0.0070 vs Vehículo.

7.3.2 Nódulos linfáticos periféricos drenantes

El análisis de este tejido permite evaluar la respuesta inmunológica a nivel regional.

Como se puede apreciar en la **figura 11**, el desarrollo tumoral modifica las subpoblaciones estudiadas, de manera que promueve la proliferación de linfocitos T citotóxicos mientras que disminuye la proporción de linfocitos T cooperadores. De igual manera, se observa un incremento en los linfocitos B como consecuencia del desarrollo del tumor.

De manera importante, el porcentaje de linfocitos T reguladores se duplica en los animales con desarrollo de tumor. Este resultado permite sugerir que el incremento de Treg en el infiltrado leucocitario tumoral tiene un componente importante de Treg naturales que proliferaron previamente en el NLP drenante, además de los Treg inducidos que pudiesen haberse generado por efecto del microambiente tumoral.

Con respecto al efecto de la disrupción endócrina neonatal, no se observan diferencias significativas en ninguna de las subpoblaciones analizadas.

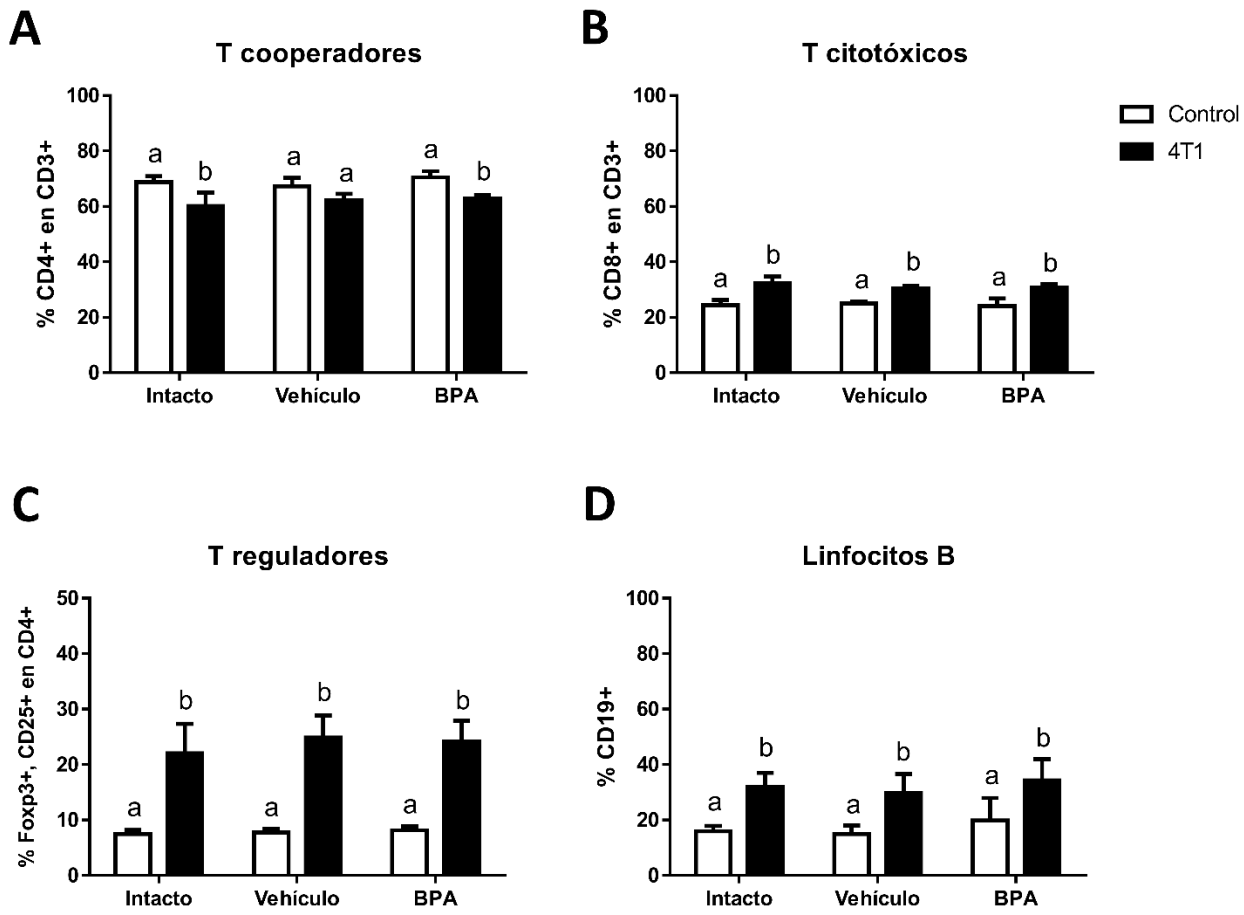

Figura 11. Análisis de las subpoblaciones inmunes en NLP drenantes. Determinación de subpoblaciones inmunológicas por citometría de flujo. **A:** linfocitos T cooperadores; **B**: linfocitos T citotóxicos; **C**: Linfocitos T reguladores; **D**: linfocitos B. Datos de 3 experimentos independientes expresados como media ± DE; n = 12 por grupo. Notación: (b ≠ a), $p < 0.05$.

7.3.3 Bazo

Al evaluar la respuesta inmunológica a nivel sistémico, en el bazo, se observaron efectos drásticos como consecuencia del desarrollo tumoral, independientes de la exposición al BPA. Llama la atención que los linfocitos T reguladores se encuentran virtualmente ausentes en este tejido en los animales a los que se les indujo el desarrollo de tumores mamarios (**fig. 12 C**). De manera análoga, los linfocitos B, las células NK y

los macrófagos se encuentran disminuidos en los animales portadores de tumor (**fig. 12 D, E y F**).

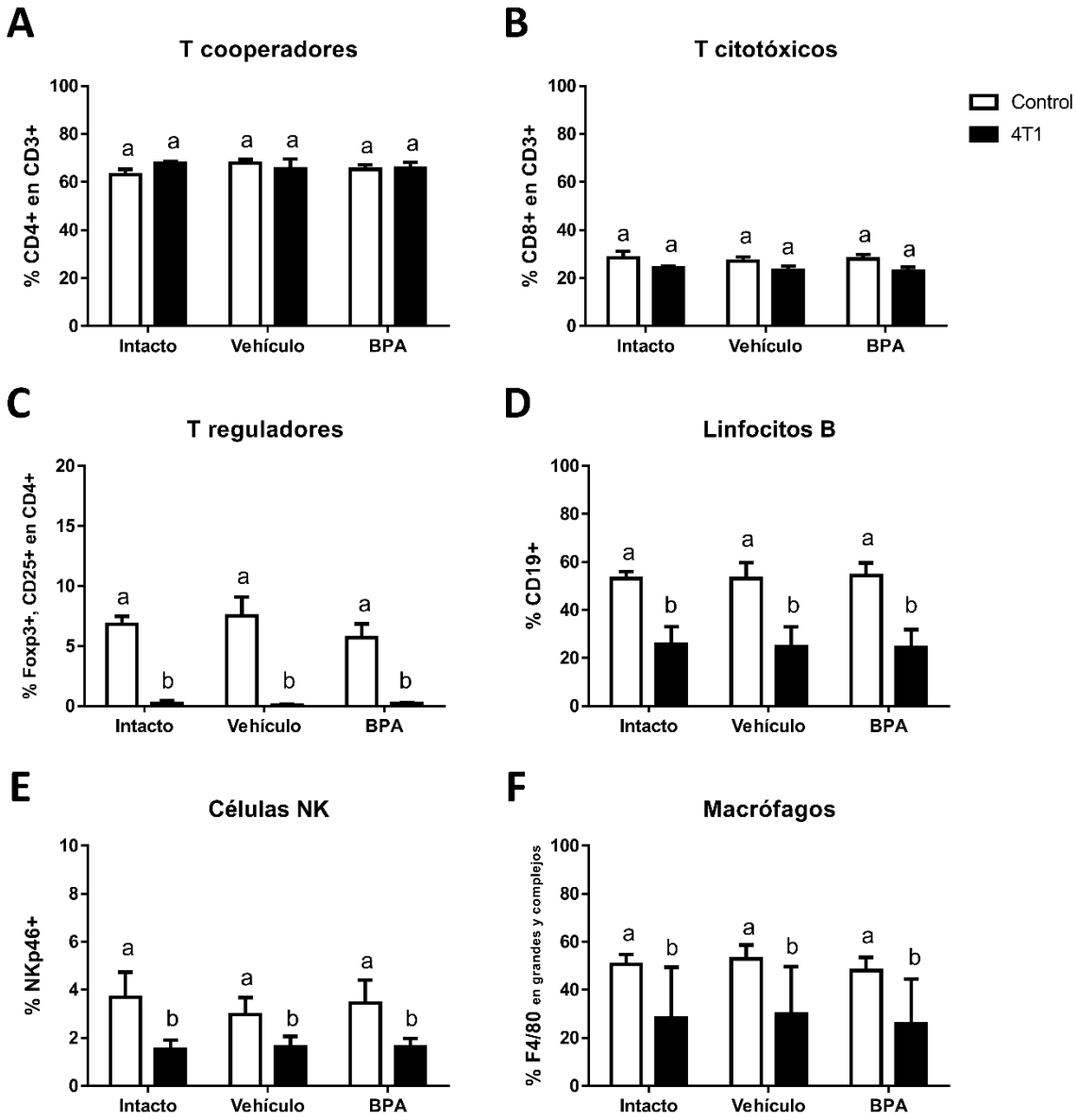

Figura 12. Análisis de las subpoblaciones inmunes en bazo. Determinación de subpoblaciones inmunológicas por citometría de flujo. **A**: linfocitos T cooperadores; **B**: linfocitos T citotóxicos; **C:** Linfocitos T reguladores; **D**: linfocitos; **E**: células NK; **F**: macrófagos. Datos de 3 experimentos independientes expresados como media ± DE; n = 12 por grupo. Notación: (b ≠ a), $p < 0.05$.

Sin embargo, no se observaron diferencias significativas por efecto del tratamiento neonatal en los grupos sanos ni en aquéllos con desarrollo de tumor.

7.4 Expresión de citocinas intratumorales

Como elemento fundamental del microambiente tumoral, se determinó la expresión de citocinas proinflamatorias (IL-1β, TNF-α e IFN-γ) y reguladoras (IL-10 y TGF-β) en el tejido tumoral, por medio de RT-PCR. Como se puede observar en la **figura 13**, el microambiente tumoral muestra una clara polarización hacia el fenotipo regulador, denotado por la predominancia de la expresión de TGF-β (**fig. 13 E**).

Sin embargo, llama la atención que el grupo expuesto a BPA, a pesar de presentar una mayor proporción de linfocitos T reguladores, no muestra una mayor expresión de TGF-β o IL-10 (**fig. 13 D, E**). No obstante, la expresión de TNF-α, así como la de IFN-γ, se encontraron disminuidas en el grupo expuesto a BPA (**fig. 13 B, C**), sugiriendo que en este grupo hay una menor respuesta proinflamatoria.

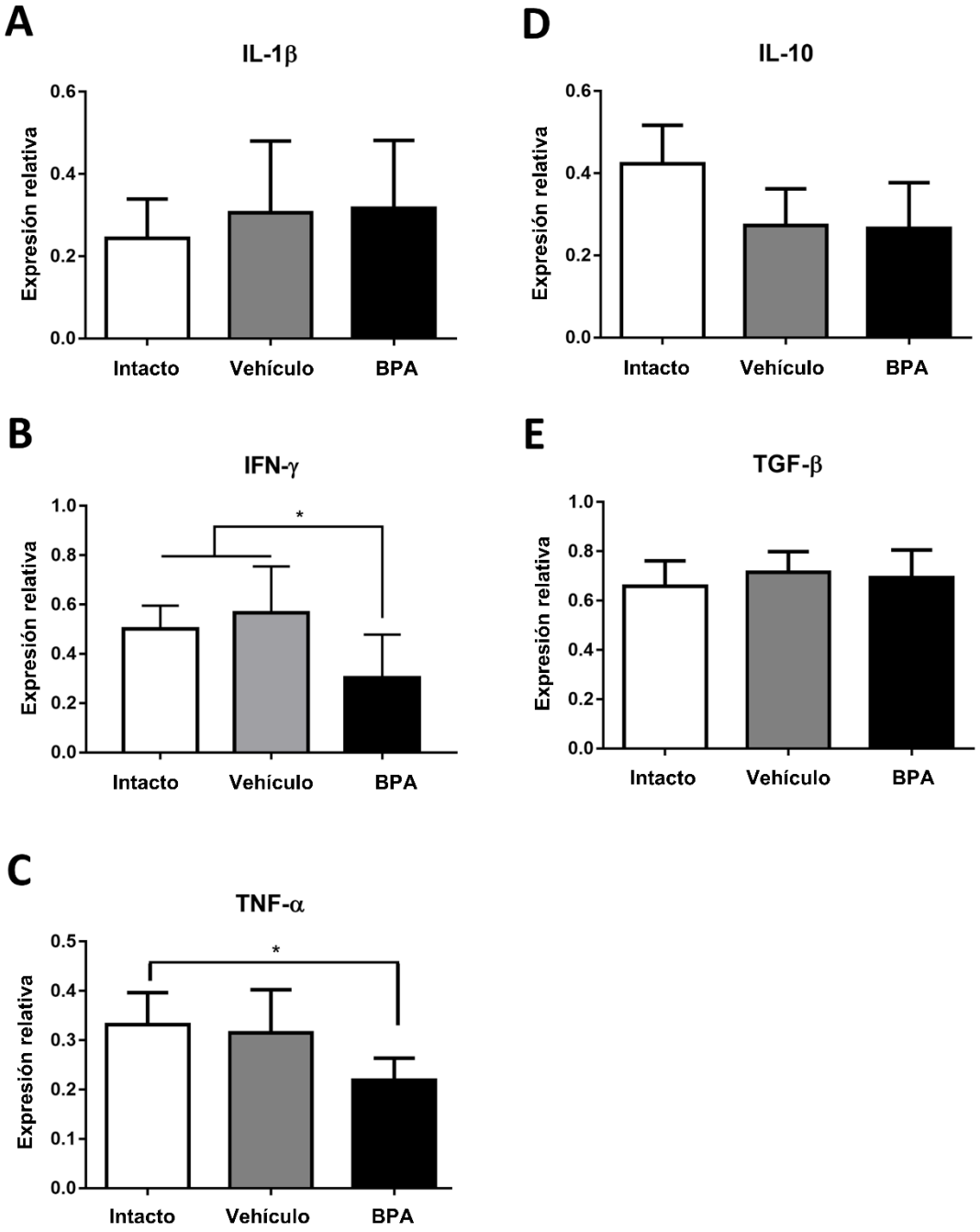

Figura 13. Expresión de citocinas intratumorales. Determinación por RT-PCR de las citocinas proinflamatorias: **A**: IL-1β, **B**: IFN-γ, **C:** TNF-α e inmunomoduladoras: **D**: IL-10 y **E**: TGF-β. Expresión normalizada respecto a la subunidad 18S ribosomal, de expresión constitutiva. Datos de 2 experimentos independientes expresados como media ± DE; n = 10 por grupo. En **B**, * p = 0.0216 vs Intacto, p = 0.0034 vs Vehículo; en **C**, * p = 0.0244 vs Intacto.

7.5 Expresión de marcadores de activación de macrófagos

Los macrófagos asociados a tumor (MATs) representan una población importante del infiltrado tumoral, en donde pueden adquirir un amplio rango de fenotipos de activación comprendido entre el de activación clásica (M1) y el de activación alternativa (M2). Por medio de RT-PCR, se evaluó la expresión de marcadores de activación clásica (iNOS) y alternativa (Arg-1, Fizz-1 y Ym-1).

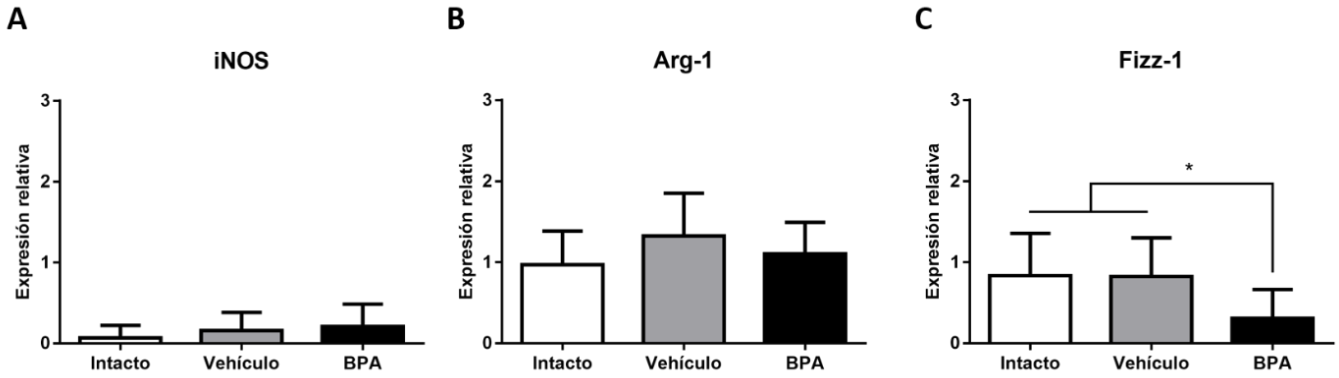

Figura 14. Perfil de activación de MATs. Expresión de marcadores de activación clásica en **A**; iNOS y alternativa en **B**: Arg-1 y **C**: Fizz-1 determinada por RT-PCR. Expresión normalizada respecto a la subunidad 18S ribosomal, de expresión constitutiva. Datos de 2 experimentos independientes expresados como media ± DE; n = 10 por grupo; * p = 0.0326 vs Intacto, p = 0.0476 vs Vehículo.

Los MATs de todos los grupos mostraron un perfil de expresión sugerente de activación alternativa, como se observa en la **figura 14**, en la que se aprecia una mayor expresión de Arg-1, con respecto a la expresión de iNOS. Sin embargo, los MATs del grupo expuesto a BPA mostraron una menor expresión de Fizz-1 (**fig. 14 C**), con una expresión normalizada promedio de 0.307 (I.C. 0.09, 0.51), comparada con un nivel de expresión de 0.834 (I.C. 0.52, 1.27) en el grupo intacto y 0.821 (I.C. 0.47, 1.26) en el vehículo.

Por otra parte, no se observó expresión del marcador Ym-1 en ninguna de las muestras analizadas.

7.6 Expresión de ERα en subpoblaciones celulares

Las células del sistema inmune están sujetas a modulación por esteroides sexuales, mediada por receptores presentes en estas células. Dado el carácter estrogénico de la disrupción endócrina causada por el BPA, se analizó la expresión del receptor ERα en las subpoblaciones celulares de interés a nivel local, regional y sistémico.

7.6.1 Infiltrado tumoral

En el microambiente tumoral, se observó un mayor porcentaje de linfocitos T con expresión francamente positiva de ERα en el grupo expuesto a BPA, en comparación con el grupo Intacto, así como un mayor nivel de expresión del mismo (**fig. 15 A**).

Los MATs infiltrados en los tumores de los individuos del grupo BPA también mostraron un mayor porcentaje de células positivas a ERα; sin embargo, el nivel de expresión en los mismos fue significativamente menor, como se aprecia en la **figura 15 B**. Este resultado podría estar relacionado con la menor expresión de Fizz-1 que presentaron los macrófagos de este grupo, pues la señalización *via* el receptor de estrógenos promueve la expresión de esta proteína [88].

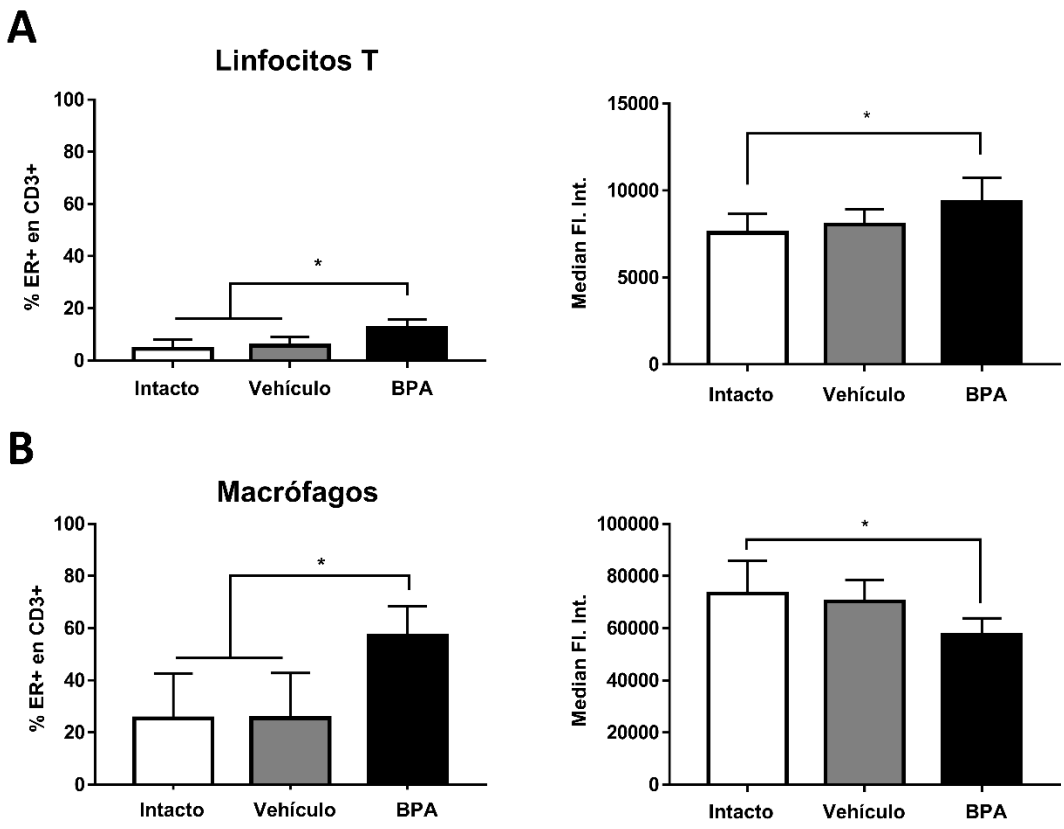

Figura 15. Evaluación de la expresión de ERα en poblaciones inmunes en el microambiente tumoral por citometría de flujo. Porcentaje de células ERα^{+} (panel izquierdo) y nivel de expresión representado por la intensidad mediana de fluorescencia (panel derecho) en **A:** linfocitos T y **B:** macrófagos. Datos de 2 experimentos independientes expresados como media ± DE; n = 10 por grupo; * $p < 0.05$ contra controles no expuestos a BPA.

7.6.2 Nódulos linfáticos drenantes

A nivel regional, se analizaron los nódulos linfáticos periféricos inguinales, los más próximos al área de desarrollo del tumor. Por la naturaleza de este tejido, sólo se analizaron poblaciones linfoides, al ser las más representadas.

En cuanto a los linfocitos T, se observó que en todas las subpoblaciones analizadas el porcentaje de positividad a ERα fue cercano al 100%, con mínima variabilidad entre los grupos experimentales (**fig. 16 A-C** panel izquierdo). Sin embargo,

es evidente que en los animales con desarrollo tumoral el nivel de expresión del receptor disminuye aproximadamente un 50% (**fig. 16 A-C,** panel derecho).

Por su parte, los linfocitos B presentaron un porcentaje de positividad ligeramente menor al de los linfocitos T, pero sin cambios entre grupos experimentales, como se muestra en el panel izquierdo de la **figura 16**. A diferencia de los linfocitos T, el nivel de expresión de ERα no se vio disminuido en los individuos con desarrollo tumoral.

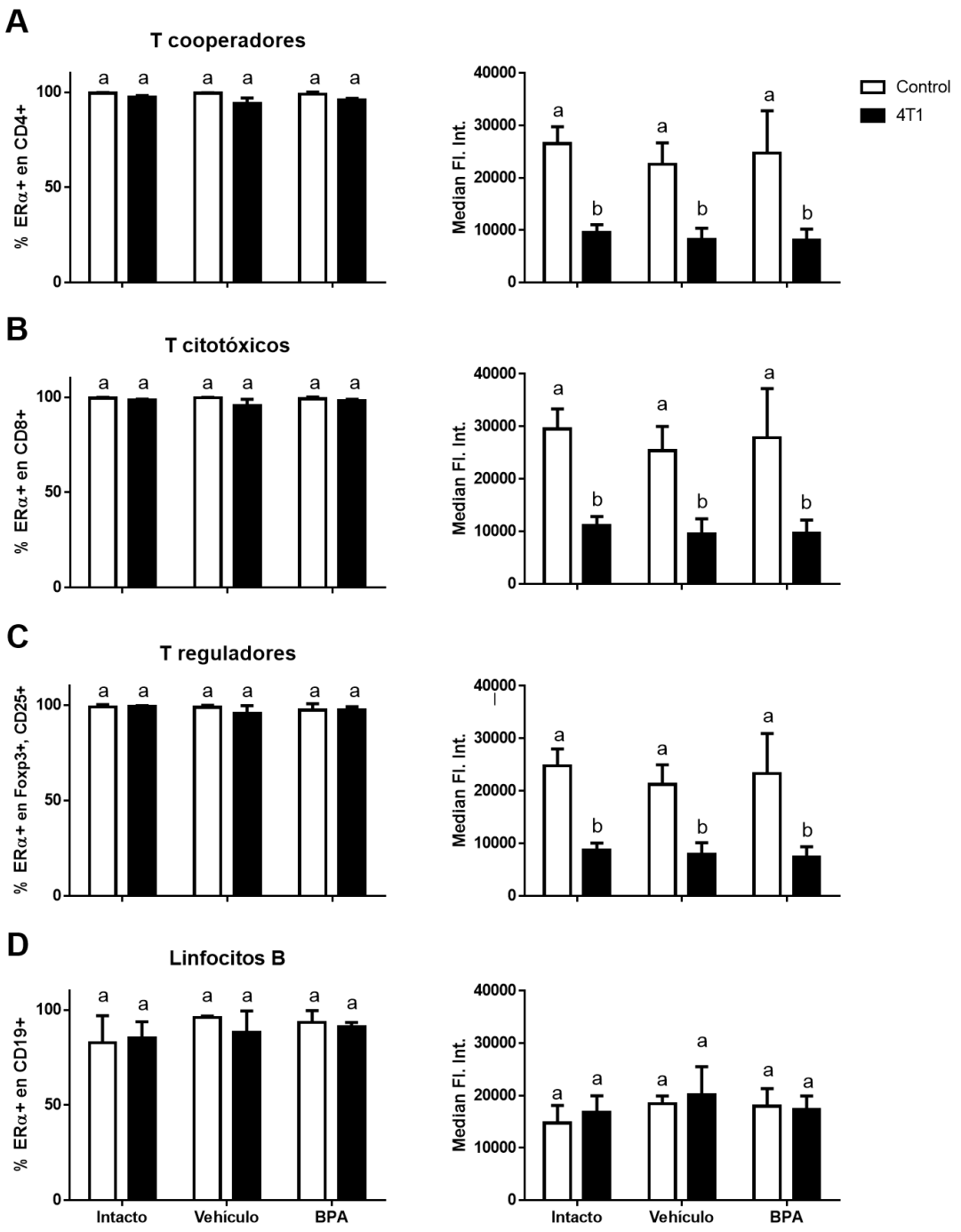

Figura 16. Evaluación de la expresión de ERα en poblaciones inmunes en NLP drenantes por citometría de flujo. Porcentaje de células ERα⁺ (panel izquierdo) y nivel de expresión representado por la intensidad mediana de fluorescencia (panel derecho). Datos de 2 experimentos independientes expresados como media ± DE; n = 10 por grupo. Notación: (b ≠ a), $p < 0.05$.

7.6.3 Bazo

A nivel sistémico, en el bazo, se observó que los linfocitos T de los animales que desarrollaron tumores presentaban una menor expresión de ERα, en comparación con los individuos sanos (**fig. 17 A-B**). Si bien este fenómeno se presentó en todos los grupos de tratamiento, éste fue más evidente en los grupos tratados con Vehículo y BPA.

Se observó un comportamiento similar en los linfocitos B y macrófagos, en los cuales la proporción de células positivas disminuyó en los animales portadores de tumor en los grupos Vehículo y BPA (**fig. 18 A y C**).

El bazo fue el único tejido en el que fue posible analizar la expresión de ERα en las células NK, principalmente porque esta población no se encuentra representada en los nódulos linfáticos y se encontraba prácticamente ausente en el microambiente tumoral en el momento en el que se realizó el análisis. El primer hallazgo fue que los bazos de los animales de los grupos 4T1 (con desarrollo tumoral) se encontraban privados de células NK, por lo que sólo fue posible evaluar la expresión de ERα en los animales de los grupos control, encontrándose que la exposición a BPA no sólo incrementó el porcentaje de células positivas, sino que éstas mostraban un mayor nivel de expresión, como se muestra en la **figura 18 B**.

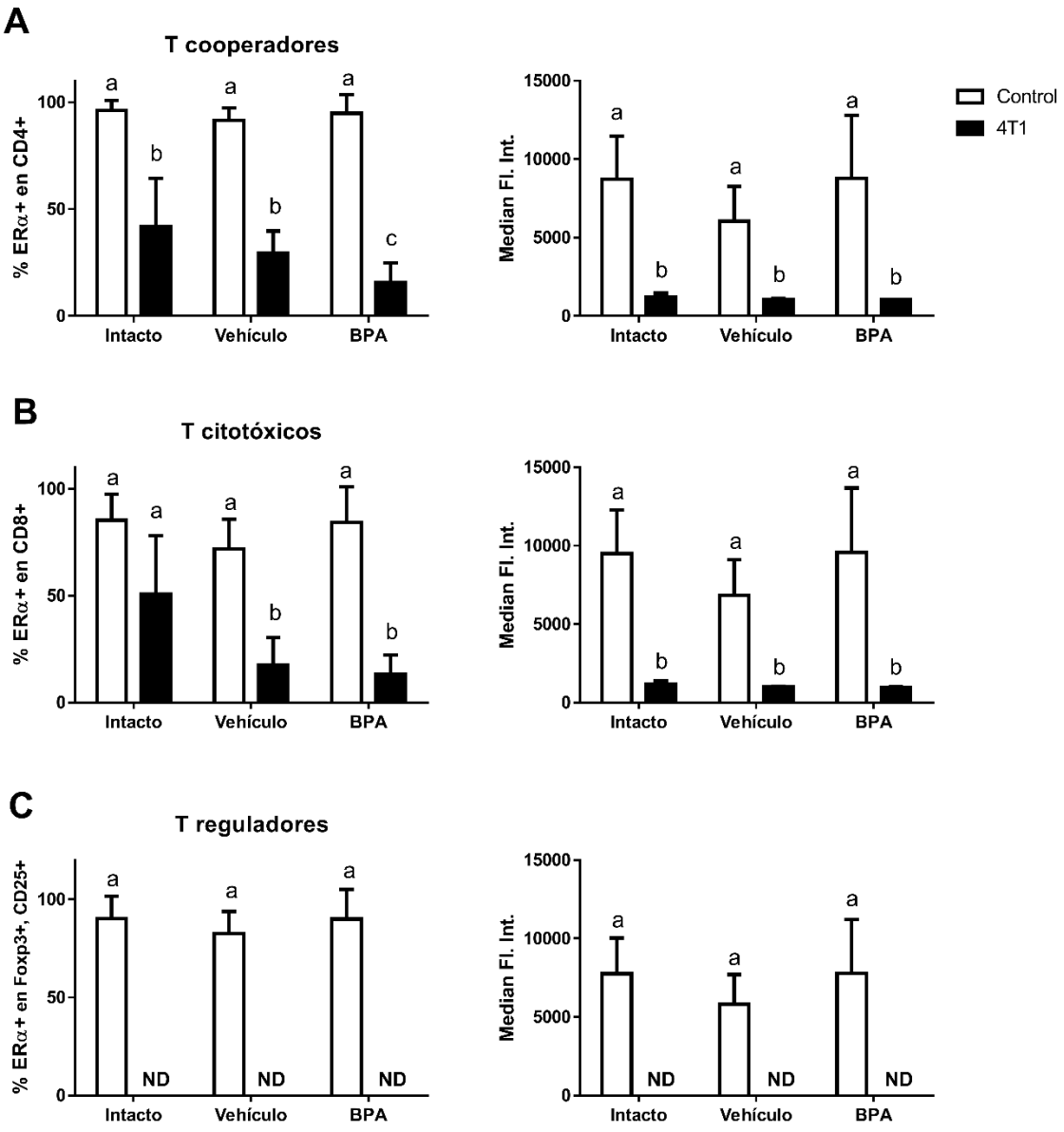

Figura 17. Evaluación de la expresión de ERα en poblaciones inmunes bazo por citometría de flujo. Porcentaje de células ERα⁺ (panel izquierdo) y nivel de expresión representado por la intensidad mediana de fluorescencia (panel derecho) en **A**: linfocitos T cooperadores, **B**: linfocitos T citotóxicos y **C**: linfocitos T reguladores. Datos de 2 experimentos independientes expresados como media ± DE; n = 10 por grupo. Notación: (b ≠ a), (c ≠ b ≠ a), $p < 0.05$; ND (datos no disponibles)**.**

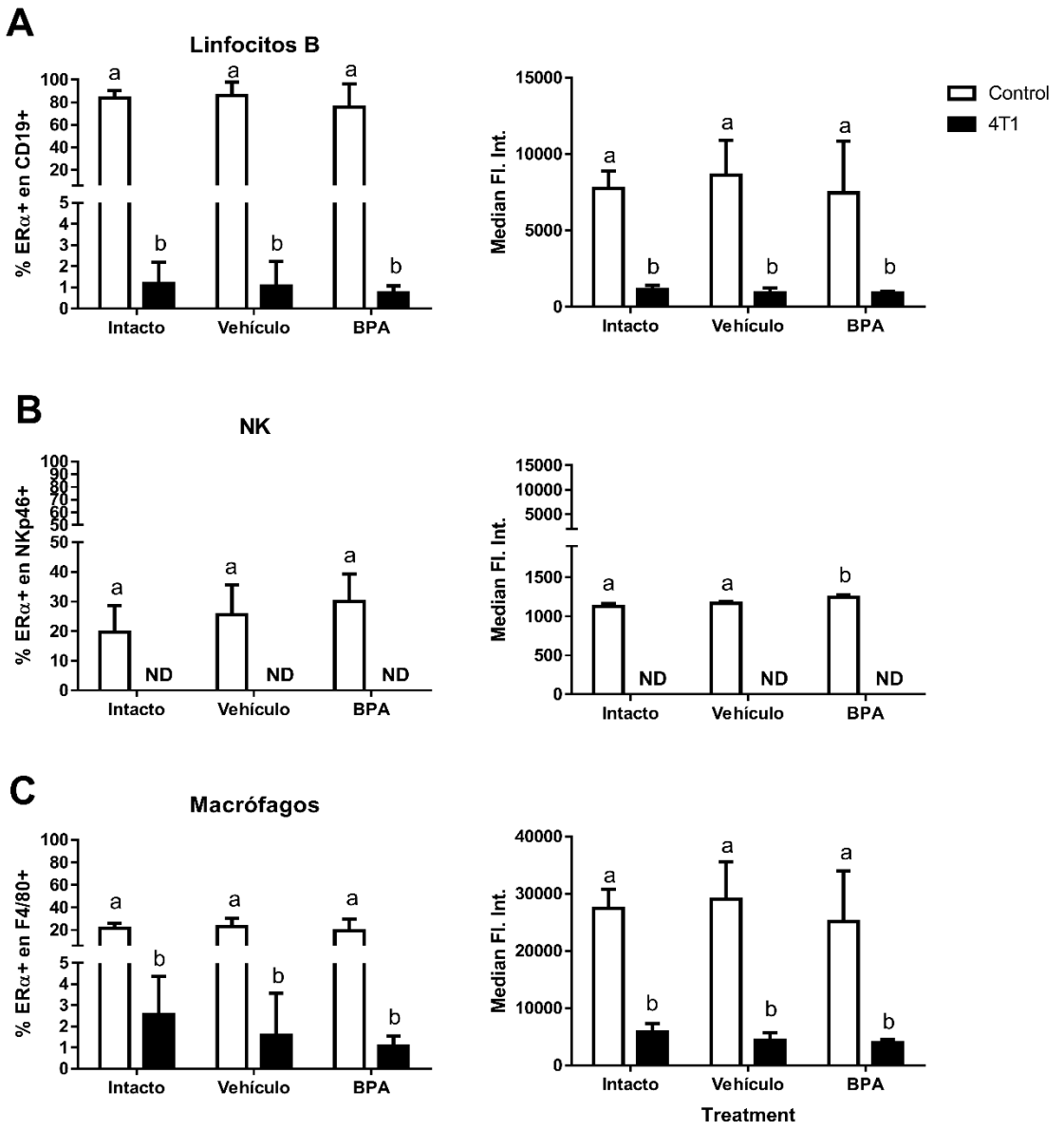

Figura 18. Evaluación de la expresión de ERα en poblaciones inmunes bazo por citometría de flujo. Porcentaje de células ERα⁺ (panel izquierdo) y nivel de expresión representado por la intensidad mediana de fluorescencia (panel derecho) en **A**: linfocitos B, **B**: células NK y **C**: macrófagos. Datos de 2 experimentos independientes expresados como media ± DE; n = 10 por grupo. Notación: (b ≠ a), $p < 0.05$; ND (datos no disponibles)**.**

Discusión

El objetivo principal de este trabajo fue evaluar las repercusiones a largo plazo de una disrupción endócrina durante el periodo neonatal sobre el sistema inmunológico, particularmente los potenciales cambios en la respuesta antitumoral.

En los roedores, la etapa crítica de desarrollo del sistema inmunológico comprende tanto la gestación como el periodo neonatal. Más allá de la formación de los órganos linfoides y la hematopoyesis, la maduración del sistema inmunológico tiene lugar entre el nacimiento y el día 30 postnatal. Además, se ha demostrado que los días 2-4 postnatales son críticos en cuanto al desarrollo de la inmunidad adaptativa mediada por linfocitos T y el desarrollo de linfocitos T reguladores, dado que la remoción quirúrgica del timo o mediante el tratamiento con ciclosporina a esta edad, pero no después, resulta en fenómenos de autoinmunidad multiorgánica [89–91].

Tomando en cuenta lo anterior, se decidió utilizar una única dosis de BPA al tercer día postnatal, ocasionando así una disrupción endócrina durante un periodo crítico en el desarrollo del sistema inmunológico.

A pesar de lo breve de la exposición, el modelo experimental que se utilizó involucraba el desarrollo de un adenocarcinoma mamario embebido en la glándula mamaria [27], lo que resulta en un sistema sensible a las hormonas. Esto supondría un sistema en el que todo cambio observado en cuanto al desarrollo tumoral podría deberse tanto a factores endócrinos –tales como mayores niveles de estradiol sérico– como a factores inmunológicos.

Originalmente se planteó el uso de la línea 4T1 como una estrategia experimental que permitiría excluir el aprovechamiento del estradiol como factor de crecimiento, dado que existen reportes que señalan que esta línea celular es un modelo de cáncer de mama triple negativo, esto es, que no expresa receptores de estrógenos, de progesterona o el receptor HER2 [28–30] y, por lo mismo, su desarrollo sería independiente de los niveles hormonales de los individuos. Sin embargo, en el laboratorio se detectó que esta línea celular sí expresa el receptor ERα. Esto planteó la posibilidad de que los cambios

observados en cuanto al desarrollo tumoral pudieran ser atribuidos tanto a factores endócrinos como a factores inmunológicos o la interacción de los mismos, ya que el ERα permite a las células aprovechar los estrógenos como factores de crecimiento.

Por lo anterior, se decidió evaluar el grado de alteración endócrina que pudiese ocurrir ante el esquema de exposición al BPA que se manejó, es decir, una única dosis de 250 µg/kg al tercer día postnatal. Si bien se encuentra bien documentado que la administración de BPA a roedores produce una variedad de efectos sobre el sistema endócrino, particularmente en el eje reproductivo, tales como inicio temprano de la pubertad, alteraciones del ciclo estral e incluso en el desarrollo de la glándula mamaria [44,45,92,93], la mayoría de estos efectos se observan tras exposiciones prolongadas en el periodo perinatal o prepuberal y a dosis mayores [92].

Como se reportó en la sección de resultados, no se observaron alteraciones en cuanto a la edad de inicio de la pubertad, la regularidad del ciclo estral ni los niveles basales de estradiol sérico, lo que nos permite sugerir que los efectos observados en cuanto al desarrollo tumoral son debidos principalmente a factores inmunológicos y no a alteraciones endócrinas significativas.

De manera sobresaliente, como consecuencia de una única exposición al BPA, se promovió el crecimiento tumoral. Estudios previos han asociado al BPA con el riesgo de desarrollar cáncer de mama, pero principalmente como consecuencia de alteraciones en el desarrollo de la glándula mamaria y una mayor susceptibilidad a la carcinogénesis [67–72]. Sin embargo, nuestros resultados muestran un nuevo aspecto mediante el cual el BPA puede contribuir al desarrollo y la progresión del cáncer de mama, pues en el modelo utilizado, la glándula mamaria del ratón no fue el objeto de la transformación celular, sino el nicho en el que se introdujeron células tumorales que se desarrollaron en mayor o menor medida dependiendo de la respuesta inmunológica generada.

Al analizar el infiltrado leucocitario de los tumores, se observó que los tumores del grupo expuesto a BPA presentaban una mayor proporción de Treg, lo cual corresponde con el mayor desarrollo de los tumores en este grupo. Se sabe que los Treg expresan las citocinas inmunomoduladoras IL-10 y TGF-β, a la vez que limitan la producción de citocinas proinflamatorias por otros tipos celulares [23,94,95]. Llamó la atención que al

analizar la expresión de citocinas intratumorales, no se observaron cambios en cuanto a la expresión de las citocinas inmunomoduladoras en el grupo expuesto a BPA. Sin embargo, este grupo sí mostró una menor expresión de las citocinas proinflamatorias IFN-γ y TNF-α, por lo que, en términos generales, el microambiente tumoral en los ratones expuestos a BPA sí presentaba un carácter menos proinflamatorio. De cualquier manera, es importante señalar que los mecanismos de regulación de los Treg no se limitan a la producción de citocinas inmunomoduladoras, sino que incluye otros aspectos que no se evaluaron, tales como la citotoxicidad e inmunosupresión por contacto y la privación de IL-2. Por otra parte, las citocinas en el microambiente tumoral se evaluaron de forma indirecta mediante RT-PCR, la cual aporta información sobre la abundancia relativa del RNA mensajero que codifica para cada proteína de interés, mas no indica la abundancia real de la proteína ya sintetizada.

Por otra parte, el microambiente tumoral no se define únicamente por las células de la inmunidad adaptativa. De hecho, los MATs constituyen una población muy importante en este microambiente. Se sabe que los MATs adquieren un fenotipo muy particular al encontrarse en el microambiente tumoral, más similar a la activación alternativa que a la activación clásica [15,96,97]. Los MATs encontrados en las muestras de tumor mostraron una muy baja expresión de iNOS y, comparativamente, una mayor expresión de Arginasa, lo que los ubica dentro del espectro de la activación alternativa. Resultó interesante que en las muestras provenientes de ratones expuestos a BPA se observó una menor expresión de Fizz-1, también conocido con RELM-α o HIMF. Aunque éste es utilizado como otro marcador de activación alternativa de macrófagos, su papel en la fisiología de estas células no está completamente elucidado. Si bien se ha planteado que puede actuar como modulador de la inflamación de tipo 2 en el pulmón [98,99], se desconocen los efectos que pueda tener en el contexto intratumoral. Por lo tanto, si bien los macrófagos en todos los grupos presentaban un perfil de activación alternativa, aquéllos infiltrados en los tumores del grupo expuesto a BPA presentaba un perfil distinto al de los presentes en los grupos control.

Es pertinente mencionar que, si bien se observan asociaciones entre el mayor desarrollo tumoral en el grupo expuesto a BPA y los cambios en las poblaciones

infiltradas en el microambiente tumoral, no es posible determinar una relación causal entre ambos. En este sentido, sería necesario estudiar las subpoblaciones a lo largo del proceso de desarrollo tumoral en un contexto libre de variables adicionales, para poder descartar que los cambios en las mismas respondieran puramente al grado de desarrollo del tumor.

Un aspecto muy importante de este trabajo fue la evaluación de la expresión del principal receptor de estrógenos, el ERα, en las células del sistema inmunológico. Como se mencionó anteriormente, las hormonas esteroides sexuales modulan la respuesta de estas células por medio de sus receptores. Los efectos que estas hormonas ejercen sobre las células inmunes son muy diversos y dependen del tipo celular, la concentración de cada hormona en particular, la combinación de las mismas e incluso las isoformas de los receptores que se expresan en un determinado momento [100–102]. Por lo anterior, incluso en el contexto de un nivel hormonal invariable, el cambio en el nivel de expresión de un receptor puede modificar la sensibilidad de una célula a una hormona en particular.

Considerando que las células inmunes expresan una variedad de receptores hormonales, no se puede sacar conclusiones definitivas basadas únicamente en la evaluación de ERα. Sin embargo, es posible hacer asociaciones entre los efectos que el principal ligando (17β-estradiol) ejerce sobre estas células y los cambios observados.

Se ha reportado, por ejemplo, que el estradiol promueve una respuesta de tipo Th2 y que altas concentraciones de esta hormona promueven la producción de IL-10, mientras que inhiben la producción de TNF-α e IFN-γ [40,103]. Considerando esto, resulta interesante que los linfocitos T del infiltrado tumoral del grupo expuesto a BPA presentaron un mayor nivel de expresión de ERα, lo cual se encuentra acorde con el hallazgo de una menor expresión de TNF-α e IFN-γ. Por otra parte, se ha reportado que los estrógenos promueven la expresión de FOXP3 (el factor de transcripción que controla la diferenciación hacia Treg) a través de ERα [104–106], lo cual podría estar relacionado con el incremento de la proporción de Treg en el microambiente tumoral, dado que los linfocitos T en este tejido mostraron un mayor nivel de expresión de ERα.

Por otra parte, se ha observado que los estrógenos son capaces de influir en la polarización de los macrófagos, promoviendo la activación alternativa durante el proceso de cicatrización cutánea [88]. Ante esto, podría establecerse una relación entre el menor grado de expresión de ERα en los macrófagos del grupo expuesto a BPA y su menor expresión de Fizz-1.

Algo muy interesante es lo que ocurre con las células NK. Estas células no fueron identificadas en el microambiente tumoral ni en los bazos de los animales que desarrollaron tumor (grupos 4T1). Sin embargo, el análisis de la expresión de ERα en estas células en los animales sanos mostró que, independientemente del desarrollo tumoral, el tratamiento neonatal con BPA causó un incremento en la expresión de ERα. Con respecto al 17β-estradiol, se sabe que altas concentraciones de esta hormona, tal como ocurre en la gestación, se asocian a una menor citotoxicidad de estas células. De tal forma, el incremento en la expresión de ERα podría resultar en una mayor sensibilidad de las células NK a las mismas concentraciones de estrógenos, lo cual podría resultar en una menor citotoxicidad de estas células en los individuos expuestos al BPA, incluso sin cambios en los niveles circulantes de estrógenos.

El hecho de que no haya sido posible analizar esta población celular en los individuos que desarrollaron tumores dificultó su estudio en este modelo en particular, dado que esta población interviene en etapas más tempranas del desarrollo tumoral. Sin embargo, ya se encuentran en proceso nuevos experimentos que permitirán evaluar esta población a tiempos más tempranos.

Conclusiones

La exposición neonatal a una única dosis de 250 µg/kg de BPA al tercer DPN no produce alteraciones evidentes en el eje reproductivo, contrario a lo que se reporta para dosis mayores y periodos de exposición más prolongados.

Este trabajo muestra que la disrupción endócrina por BPA ocurrida durante un periodo crítico de desarrollo favorece un mayor desarrollo tumoral en la vida adulta, el cual está asociado a alteraciones en la respuesta inmune. Esta disparidad se caracteriza por una mayor proporción de linfocitos T reguladores infiltrados en el microambiente tumoral, menor expresión de citocinas proinflamatorias y cambios en el perfil de activación de los macrófagos asociados a tumor.

Dado que la exposición a BPA modificó los patrones de expresión del receptor ERα en las células del sistema inmunológico y considerando que este es uno de los principales mecanismos de regulación de estas células dentro de la red neuroinmunoendocrina, es posible esta sea una de las causas subyacentes de las alteraciones observadas.

Referencias

1. Secretaría de Salud. *Compendio de patología mamaria*. (Secretaría de Salud, 2002).
2. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerCase No. 11*. (2013).
3. INEGI. *Estadísticas a propósito del día mundial de la lucha contra el cáncer de mama (19 de octubre)*. (2016).
4. Burnet, S. M. Cancer - A biological approach. *Br. Med. J.* 779–786 (1957).
5. Dunn, G. P. *et al.* The immunobiology of Cancer Immunosurveillance and Immunoediting. *Immunity* **21,** 137–148 (2004).
6. Smyth, M. J., Dunn, G. P. & Schreiber, R. D. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv. Immunol.* **90,** 1–50 (2006).
7. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting : from immuno- surveillance to tumor escape. **3,** 991–998 (2002).
8. Nava-Castro, K. E. *et al.* The immunoendocrine network in breast cancer. *Adv. Neuroimmune Biol.* **5,** 109–131 (2014).
9. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat. Immunol.* **9,** 503–10 (2008).
10. Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356,** 1795–1799 (2000).
11. Garrido, F., Cabrera, T. & Aptsiauri, N. 'Hard' and 'soft' lesions underlying the HLA class I alterations in cancer cells: Implications for immunotherapy. *Int. J. Cancer* **127,** n/a-n/a (2010).
12. Bottino, C., Castriconi, R., Moretta, L. & Moretta, A. Cellular ligands of activating NK receptors. *Trends Immunol.* **26,** 221–226 (2005).
13. Mamessier, E., Bertucci, F., Sabatier, R., Birnbaum, D. & Olive, D. 'Stealth' tumors: Breast cancer cells shun NK-cells anti-tumor immunity. *Oncoimmunology* **1,** 366–368 (2012).
14. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization : tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. **23,** 549–555 (2002).
15. Murray, P. J. & Wynn, T. a. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* **11,** 723–37 (2011).

16. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* **11,** 889–96 (2010).

17. Ruffell, B., Affara, N. I. & Coussens, L. M. Differential macrophage programming in the tumor microenvironment. *Trends Immunol.* **33,** 119–26 (2012).

18. Brady, N. J., Chuntova, P. & Schwertfeger, K. L. Macrophages: Regulators of the Inflammatory Microenvironment during Mammary Gland Development and Breast Cancer. *Mediators Inflamm.* **2016,** 4549676 (2016).

19. Hao, N.-B. *et al.* Macrophages in tumor microenvironments and the progression of tumors. *Clin. Dev. Immunol.* **2012,** 948098 (2012).

20. Dirkx, A. E. M., Oude Egbrink, M. G. a, Wagstaff, J. & Griffioen, A. W. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J. Leukoc. Biol.* **80,** 1183–96 (2006).

21. Egilmez, N. K. & Kilinc, M. O. Tumor-Resident CD8+ T-cell: The Critical Catalyst in IL-12-Mediated Reversal of Tumor Immune Suppression. *Arch. Immunol. Ther. Exp. (Warsz).* **58,** 399–405 (2010).

22. Kennedy, R. & Celis, E. Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunol. Rev.* **222,** 129–144 (2008).

23. Shevach, E. M. *et al.* The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol. Rev.* **212,** 60–73 (2006).

24. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* **4,** 330–336 (2003).

25. Nishikawa, H. & Sakaguchi, S. Regulatory T cells in tumor immunity. *Int. J. Cancer* **127,** n/a-n/a (2010).

26. Cardiff, R. D. *Comparative pathobiology of breast cancer*. (IOS Press, 2007).

27. Pulaski, B. A. & Ostrand-Rosenberg, S. Mouse 4T1 breast tumor model. *Curr. Protoc. Immunol.* **Chapter 20,** Unit 20.2 (2001).

28. Yang, X. *et al.* Estradiol increases ER-negative breast cancer metastasis in an experimental model. *Clin. Exp. Metastasis* **30,** 711–21 (2013).

29. Sztalmachova, M. *et al. International journal of oncology. International Journal of Oncology* **46,** (University of Crete, Faculty of Medicine, Laboratory of Clinical Virology, 2015).

30. Bouchard, G. *et al.* Stimulation of triple negative breast cancer cell migration and metastases formation is prevented by chloroquine in a pre-irradiated mouse model. *BMC Cancer* **16,** 361 (2016).

31. ATCC. *Q&A ATCC Breast Cancer Research Resources*.

32. BESEDOVSKY, H. O. & REY, A. DEL. Immune-Neuro-Endocrine Interactions: Facts and Hypotheses. *Endocr. Rev.* **17,** 64–102 (1996).

33. Arteaga, M., Chavarría, A. & Morales Montor, J. Immunoneuroendocrine communication network and homeostasis regulation: the use of hormones and neurohormones as immunotherapy-. *Rev. Invest. Clin.* **54,** 542–9 (2002).

34. Besedovsky, H. O. & del Rey, A. The cytokine-HPA axis feed-back circuit. *Z. Rheumatol.* **59 Suppl 2,** II/26-30 (2000).

35. Grossman, C. J. & Roselle, G. A. The interrelationship of the HPG-thymic axis and immune system regulation. *J. Steroid Biochem.* **19,** 461–7 (1983).

36. Jacobson, D. L., Gange, S. J., Rose, N. R. & Graham, N. M. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin. Immunol. Immunopathol.* **84,** 223–43 (1997).

37. Cooper, G. S. & Stroehla, B. C. The epidemiology of autoimmune diseases. *Autoimmun. Rev.* **2,** 119–25 (2003).

38. Da Silva, J. A. Sex hormones and glucocorticoids: interactions with the immune system. *Ann. N. Y. Acad. Sci.* **876,** 102-17–8 (1999).

39. Bebo, B. F. *et al.* Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. *J. Immunol.* **166,** 2080–9 (2001).

40. Muñoz-Cruz, S., Togno-Pierce, C. & Morales-Montor, J. Non-Reproductive Effects of Sex Steroids : Their Immunoregulatory Role . Or The Regulatory Role of Sex Steroids on Innate and Adaptive Immunity. *Curr. Top. Med. Chem.* **11,** (2011).

41. Diamanti-Kandarakis, E. *et al.* Endocrine-Disrupting Chemicals. *Endocr. Rev.* **30,** 293–342 (2009).

42. Ben Rhouma, K., Tébourbi, O., Krichah, R. & Sakly, M. Reproductive toxicity of DDT in adult male rats. *Hum. Exp. Toxicol.* **20,** 393–397 (2001).

43. Honma, S. *et al.* Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. *Reprod. Toxicol. Elmsford NY* **16,** 117–122 (2002).

44. Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G. & Vom Saal, F. S. Exposure to bisphenol A advances puberty. *Nature* **401,** 763–764 (1999).

45. Nah, W. H., Park, M. J. & Gye, M. C. Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and estrous cycle in female mice. *Clin. Exp. Reprod. Med.* **38,** 75–81 (2011).

46. Timms, B. G. *et al.* Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 7014–7019 (2005).

47. Gupta, C. Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. *Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med.*

New York NY **224,** 61–68 (2000).

48. Parks, L. G. *et al.* The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci. an Off. J. Soc. Toxicol.* **58,** 339–349 (2000).

49. Richter, C. A. *et al.* In Vivo Effects of Bisphenol A in Laboratory Rodent Studies. *Reprod. Toxicol.* **24,** 199–224 (2008).

50. Cimafranca, M. a *et al.* Acute and chronic effects of oral genistein administration in neonatal mice. *Biol. Reprod.* **83,** 114–21 (2010).

51. Markey, C. M., Coombs, M. a, Sonnenschein, C. & Soto, A. M. Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. *Evol. Dev.* **5,** 67–75 (2003).

52. Wetherill, Y. B. *et al.* In vitro molecular mechanisms of bisphenol A action. *Reprod. Toxicol.* **24,** 178–198 (2007).

53. Matthews, J. B., Twomey, K. & Zacharewski, T. R. In Vitro and in Vivo Interactions of Bisphenol A and Its Metabolite, Bisphenol A Glucuronide, with Estrogen Receptors a and b. *Chem. Res. Toxicol.* **14,** (2001).

54. Gutendorf, B. & Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **166,** 79–89 (2001).

55. Rich, R. L. *et al.* Kinetic analysis of estrogen receptor/ligand interactions. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 8562–7 (2002).

56. Takayanagi, S. *et al.* Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. *Toxicol. Lett.* **167,** 95–105 (2006).

57. Matsushima, A. *et al.* Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. *J. Biochem.* **142,** 517–24 (2007).

58. Alonso-Magdalena, P. *et al.* Bisphenol-A acts as a potent estrogen via non-classical estrogen triggered pathways. *Mol. Cell. Endocrinol.* **355,** 201–7 (2012).

59. Goodson, A., Robin, H., Summerfield, W. & Cooper, I. Migration of Bisphenol A from can coatings — effects of damage , storage conditions and heating. *Food Addit. Contam.* **21,** 1015–1026 (2004).

60. Le, H. H., Carlson, E. M., Chua, J. P. & Belcher, S. M. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. *Toxicol. Lett.* **176,** 149–156 (2008).

61. Fleisch, A. F., Sheffield, P. E., Chinn, C., Edelstein, B. L. & Philip, J. Bisphenol A and Related Compounds in Dental Materials. *Pediatrics* **126,** 760–768 (2010).

62. Kingman, A. *et al.* Bisphenol a and other compounds in human saliva and urine

associated with the placement of composite restorations. *J. Am. Dent. Assoc.* **143,** 1292–1302 (2012).

63. Vandenberg, L. N., Hauser, R., Marcus, M., Olea, N. & Welshons, W. V. Human exposure to bisphenol A (BPA). *Reprod. Toxicol.* **24,** 139–77 (2007).

64. Kang, J., Kondo, F. & Katayama, Y. Human exposure to bisphenol A. *Toxicology* **226,** 79–89 (2006).

65. Bailey, A., Hatwell, K. & Mihalov, J. *Exposure to Bisphenol A (BPA) for infants, toddlers and adults from the consumption of infant formula, toddler food and adult (canned) food.* (2009).

66. Palmer, J. R. *et al.* Risk of breast cancer in women exposed to diethylstilbestrol in utero: prelimiinary results (United States). *Cancer Causes Control* **13,** 753–8 (2002).

67. Yang, L. *et al.* Effect of low dose bisphenol A on the early differentiation of human embryonic stem cells into mammary epithelial cells. *Toxicol. Lett.* **218,** 187–93 (2013).

68. Vandenberg, L. N. *et al.* Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. *Endocrinology* **148,** 116–27 (2007).

69. Muñoz-de-Toro, M. *et al.* Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. *Endocrinology* **146,** 4138–47 (2005).

70. Tharp, A. P. *et al.* Bisphenol A alters the development of the rhesus monkey mammary gland. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 8190–5 (2012).

71. Murray, T. J., Maffini, M. V, Ucci, A. a, Sonnenschein, C. & Soto, A. M. Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. *Reprod. Toxicol.* **23,** 383–90 (2007).

72. Durando, M. *et al.* Prenatal Bisphenol A Exposure Induces Preneoplastic Lesions in the Mammary Gland in Wistar Rats. *Environ. Health Perspect.* **115,** 80–86 (2006).

73. Yoshitake, J. *et al.* Suppression of NO production and 8-nitroguanosine formation by phenol-containing endocrine-disrupting chemicals in LPS-stimulated macrophages: involvement of estrogen receptor-dependent or -independent pathways. *Nitric Oxide* **18,** 223–8 (2008).

74. Young Kim, J. & Gwang Jeong, H. Down-regulation of inducible nitric oxide synthase and tumor necrosis factor-α expression by bisphenol A via nuclear factor-κB inactivation in macrophages. *Cancer Lett.* **196,** 69–76 (2003).

75. Holladay, S. D. *et al.* Perinatal bisphenol A exposure in C57B6/129svj male mice: potential altered cytokine/chemokine production in adulthood. *Int. J. Environ. Res. Public Health* **7,** 2845–52 (2010).

76. Yoshino, S. *et al.* Effects of bisphenol A on antigen-specific antibody production, proliferative responses of lymphoid cells, and TH1 and TH2 immune responses in mice. *Br. J. Pharmacol.* **138,** 1271–6 (2003).

77. Lee, M. H. *et al.* Enhanced interleukin-4 production in CD4+ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: involvement of nuclear factor-AT and Ca2+. *Immunology* **109,** 76–86 (2003).

78. Yurino, H. *et al.* Endocrine disruptors (environmental estrogens) enhance autoantibody production by B1 cells. *Toxicol. Sci.* **81,** 139–47 (2004).

79. Pisapia, L. *et al.* General and Comparative Endocrinology Effects of some endocrine disruptors on cell cycle progression and murine dendritic cell differentiation. **178,** 54–63 (2012).

80. Watanabe, H. *et al.* Bisphenol A significantly enhances the neutrophilic differentiation of promyelocytic HL-60 cells. *Int. Immunopharmacol.* **3,** 1601–8 (2003).

81. Tian, J. *et al.* Effects of bisphenol A on the development of central immune organs of specific-pathogen-free chick embryos. *Toxicol. Ind. Health* (2012). doi:10.1177/0748233712452776

82. Guzmán, C., Camacho-Arroyo, I., De León-Nava, M. a & Morales-Montor, J. Neonatal exposure to estradiol induces resistance to helminth infection and changes in the expression of sex steroid hormone receptors in the brain and spleen in adult mice of both sexes. *Brain. Behav. Immun.* **23,** 709–15 (2009).

83. Khan, D. & Ahmed, S. A. Epigenetic Regulation of Non-Lymphoid Cells by Bisphenol A, a Model Endocrine Disrupter: Potential Implications for Immunoregulation. *Front. Endocrinol. (Lausanne).* **6,** 91 (2015).

84. Doshi, T., Mehta, S. S., Dighe, V., Balasinor, N. & Vanage, G. Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. *Toxicology* **289,** 74–82 (2011).

85. Santangeli, S. *et al.* BPA-Induced Deregulation Of Epigenetic Patterns: Effects On Female Zebrafish Reproduction. *Sci. Rep.* **6,** 21982 (2016).

86. Kundakovic, M. *et al.* Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 9956–61 (2013).

87. Kumar, D. & Thakur, M. K. Effect of perinatal exposure to Bisphenol-A on DNA methylation and histone acetylation in cerebral cortex and hippocampus of postnatal male mice. *J. Toxicol. Sci.* **42,** 281–289 (2017).

88. Campbell, L. *et al.* Estrogen Receptor-Alpha Promotes Alternative Macrophage Activation during Cutaneous Repair. *J. Invest. Dermatol.* **134,** 2447–2457 (2014).

89. Sakaguchi, S., Takahashi, T. & Nishizuka, Y. Study on cellular events in

postthymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J. Exp. Med.* **156,** (1982).

90. Bonomo, A., Kehn, P. J. & Shevach, E. M. Post-thymectomy autoimmunity: abnormal T-cell homeostasis. *Immunol. Today* **16,** 61–67 (1995).

91. Classen, J. B. Cyclosporine Induced Autoimmunity in Newborns Prevented by Early Immunization. *Autoimmunity* **27,** 135–139 (1998).

92. Rasier, G., Toppari, J., Parent, a-S. & Bourguignon, J.-P. Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: a review of rodent and human data. *Mol. Cell. Endocrinol.* **254–255,** 187–201 (2006).

93. Dhimolea, E. *et al.* Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the propensity to neoplastic development. *PLoS One* **9,** e99800 (2014).

94. Wang, H. Y. & Wang, R.-F. Regulatory T cells and cancer. *Curr. Opin. Immunol.* **19,** 217–23 (2007).

95. Ghiringhelli, F., Ménard, C., Martin, F. & Zitvogel, L. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunol. Rev.* **214,** 229–38 (2006).

96. Qian, B.-Z. & Pollard, J. W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141,** 39–51 (2010).

97. Mantovani, A., Sica, A., Allavena, P., Garlanda, C. & Locati, M. Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum. Immunol.* **70,** 325–30 (2009).

98. Yamaji-Kegan, K. *et al.* Hypoxia-Induced Mitogenic Factor (HIMF/FIZZ1/RELM) Increases Lung Inflammation and Activates Pulmonary Microvascular Endothelial Cells via an IL-4-Dependent Mechanism. *J. Immunol.* **185,** 5539–5548 (2010).

99. Nair, M. G. *et al.* Alternatively activated macrophage-derived RELM-{alpha} is a negative regulator of type 2 inflammation in the lung. *J. Exp. Med.* **206,** 937–52 (2009).

100. Nilsson, S., Ma, S., Treuter, E., Tujague, M. & Thomsen, J. Mechanisms of Estrogen Action. **81,** (2001).

101. Diel, P. Tissue-specific estrogenic response and molecular mechanisms. *Toxicol. Lett.* **127,** 217–24 (2002).

102. Filardo, E. J. & Thomas, P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology* **153,** 2953–62 (2012).

103. Hernández-Cervantes, R., Sánchez-Acosta, A. G., Morales-Montor, J. & Ricardo, R.-N. Regulación neuroendocrinológica de la función inmunitaria: el papel de la

hipófisis y los esteroides sexuales. *Rev. Espec. en Ciencias Químico-Biológicas* **13,** 103–112 (2010).

104. Polanczyk, M. J. *et al.* Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. *J. Immunol.* **173,** 2227–2230 (2004).

105. Ohkura, N., Hamaguchi, M. & Sakaguchi, S. FOXP3+ regulatory T cells: control of FOXP3 expression by pharmacological agents. *Trends Pharmacol. Sci.* **32,** 158–66 (2011).

106. Tai, P. *et al.* Induction of regulatory T cells by physiological level estrogen. *J. Cell. Physiol.* **214,** 456–464 (2008).

Anexo I

Artículo original

SCIENTIFIC REPORTS

OPEN

A single neonatal administration of Bisphenol A induces higher tumour weight associated to changes in tumour microenvironment in the adulthood

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BPA is an oestrogenic endocrine disrupting chemical compound. Exposure to BPA in as early as pregnancy leads to lifelong effects. Since endocrine and immune systems interact in a bidirectional manner, endocrine disruption may cause permanent alterations of the immune system, affecting a future anti-tumoral response. Neonate (PND 3) female syngeneic BALB/c mice were exposed to a single dose of 250 µg/kg BPA. Once sexual maturity was reached, a mammary tumour was induced injecting 4T1 cells *in situ*, these cells are derived from a spontaneous adenocarcinoma in a BALB/c mouse and therefore allows for an immunocompetent recipient. After 25 days of injection, showing no major endocrine alterations, BPA-exposed mice developed larger tumours. Tumour leukocytic infiltrate analysis revealed a higher proportion of regulatory T lymphocytes in the BPA-exposed group. RT-PCR analysis of tumour samples showed a decreased expression of TNF-α and IFN-γ, as well as the M2 macrophage marker Fizz-1 in the BPA-exposed group. Flow cytometry analysis revealed differences in ERα expression by T lymphocytes, macrophages and NK cells, both associated to BPA exposure and tumour development. These findings show a new aspect whereby early life BPA exposure can contribute to breast cancer development and progression by modulating the anti-tumoral immune response.

Human activity has led to an increased amount of environmental contaminants, including endocrine disrupting chemicals (EDCs). EDCs are defined as exogenous compounds interfering with the synthesis, secretion, metabolism, transport, mechanism of action, and elimination of endogenous steroid hormones[1]. Bisphenol A (BPA), an oestrogenic EDC, is characterized by its high affinity to nuclear oestrogen receptors (ERα and ERβ)[2,3]. Moreover, BPA is widely used in the production of polycarbonate plastics, epoxy resins (used in food containers and can linings, respectively), as well as dental sealants; all of them used daily by people. BPA can leak from these materials when exposed to high temperatures, acidic conditions, or saliva, thus leading to human exposure[4–6].

BPA causes several endocrine alterations, not only in the reproductive organs[7–11] but also in mammary gland development[12–17], and brain sexual differentiation[18–20]. The nature and magnitude of these effects depends on the dose, exposure course, and the developmental stage at which the exposure occurs. Regarding the latter, endocrine disruption during critical developmental stages could lead to lifelong effects.

It is well known that BPA exposure can occur as early as during gestation, as evidenced by reports of BPA presence in amniotic fluid, foetal serum, and breast milk[21,22]. In this regard, there is an existing concern about the effects that BPA could exert in a developing organism, including the immune system.

Concerning the immune system in mice, the critical developmental stages encompass both the gestational and neonatal periods. Beyond the immune organ formation and hematopoiesis, the maturation of the immune

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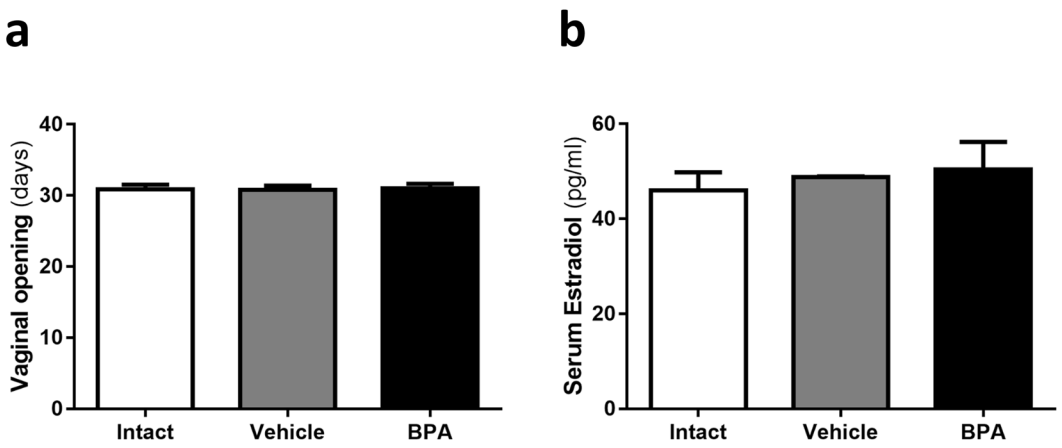

Figure 1. Endocrine parameters assessment. (**a**) Vaginal opening as puberty onset indicator. (**b**) Serum 17β-Oestradiol levels at diestrus phase at 12 weeks of age measured by EIA. Data from 2 independent experiments are expressed as mean ± SD; n = 14 in (**a**) and 8 in (**b**).

system occurs between birth and post natal day 30(PND 30)[23]. Moreover, it has been demonstrated that PND 2–4 are critical for T lymphocyte development, since thymectomy or cyclosporine treatment at this age, but not later, results in multi-organ autoimmunity[24–26].

On the other hand, cancer is the second cause of death worldwide; according to the WHO[27], it accounted for 8.8 million deaths in 2015. Breast cancer is among the most prevalent types of cancer, and several environmental factors have been linked to an increased incidence of this ailment. When talking about cancer, there are multiple factors that should be taken into account, not only those contributing to cellular stress, DNA damage, and emergence of transformed cells, but also the mechanisms responsible for the detection and elimination of cancer cells. In this context, the immune system, particularly cell populations such as NK cells, T lymphocytes, regulatory T lymphocytes (Treg), and Tumour Associated Macrophages (TAMs), as well as their secreted cytokines, play a major role in preventing cancer development or controlling its progression[28,29].

The endocrine and immune systems are not independent physiological entities, but parts of a net of bidirectional interactions mediated by hormones, cytokines and even neurotransmitters which have been widely documented in recent years[30,31]. Regarding sexual steroid hormones, they regulate the distribution and functionality of several immune cell populations, modulating the immune response, maturation and selection of thymocytes, lymphoid proliferation, and cytokine production[31]. The modulatory effects of sex steroids are mediated by specific receptors present in immune cells, namely oestrogen receptors (ERα, ERβ), progesterone receptor (PR), and androgen receptor (AR), as well as membrane oestrogen and progesterone receptors, among others[31].

Since sexual steroids modulate the immune system, exogenous agents such as EDCs could also influence it.

Therefore, we decided to assess the potential alteration of the anti-tumoral immune response caused by BPA exposure at a critical developmental stage, specifically during the neonatal period. Our results clearly demonstrate that BPA administered during the neonatal period has an impact on tumour size, weight, and immune-endocrine microenvironment during adult life.

Results

Endocrine Parameters. The experimental strategy involves the development of a tumour within hormone-sensitive tissue; therefore, it was important to discriminate whether the observed effects could be attributed to the direct or indirect effect of neonatal endocrine disruption on the immune system or caused by a persistent hormonal alteration.

In order to assess the potential reproductive effects of a single 250 μg/kg bw BPA dose, puberty onset was determined by the age of vaginal opening. Contrary to what has been reported with higher or more prolonged exposures, neonatal BPA exposure did not alter puberty onset (Fig. 1a)[8,9].

In addition, the oestrous cycle was also monitored once sexual maturity was fully established (8 weeks old), finding no difference between the experimental groups (data not shown). Furthermore, BPA exposure had no effect on baseline levels of serum oestradiol during the diestrus phase (Fig. 1b).

Tumour size and weight. A significant promotion of tumour development was observed in the BPA-exposed group. At 25 days after the inoculation of tumour cells, it was evident that the mice subjected to neonatal BPA exposure developed larger tumours (Fig. 2a). Indeed, after measuring tumour weight, those found in BPA-exposed mice showed an 88% increase in weight when compared to unexposed groups (Fig. 2b), *i.e.* in the BPA group, the mean relative weight was 1.88 (CI 1.48, 2.28) compared to a mean relative weight of 1 (CI 0.8, 1.19) for the Intact group and a mean relative weight of 1.12 (CI 0.98, 1.25) for the Vehicle group.

Tumour infiltrate. The flow cytometry analysis of leukocytic infiltrate in tumours revealed that the cellular composition was mostly similar between the groups (Fig. 3a–c); however, Treg were increased in the BPA-exposed

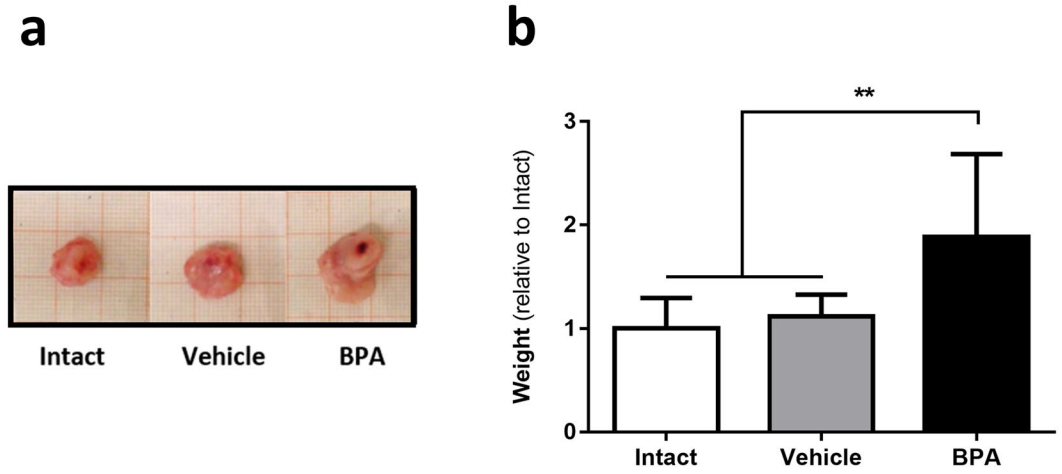

Figure 2. Tumour growth. (**a**) Representative images of tumours of the three experimental groups; millimetric grid as background. (**b**) Evaluation of final weight after 25 days of tumour development; data from 3 independent experiments are expressed as mean ± SD; n = 12; $^{**}p = 0.007$ vs Intact, $p = 0.0025$ vs Vehicle.

group (Fig. 3d,e), comprising a 13.93% (CI 10.06, 17.79) of CD4+ lymphocytes when compared to 7.82% (CI 5.77, 9.87) and 7.33% (CI 3.81, 10.85) in intact and vehicle groups, respectively.

Further, immunofluorescence analysis of tumour sections confirmed the greater proportion of regulatory T lymphocytes within the tumours of BPA-exposed mice (Fig. 4), comprised by an average of 42.8% (CI 30.27, 55.33) of CD4 + cells, compared to 21.54% (CI 14.14, 28.94) in the intact group and 21.88% (CI 11.52, 30.64) in the vehicle group.

Tumour associated macrophages (TAMs) represent an important subset of leukocytic infiltrate in many kinds of tumours, where they may display a classical (M1) or an alternative (M2) activation phenotype. By means of RT-PCR, the relative expression of classical (iNOS) and alternative (Arginase, Fizz-1 and Ym-1) activation markers was assessed. TAMs of all groups were clearly activated in an alternative manner, as evidenced by a higher arginase expression when compared to iNOS (Fig. 5a,c). However, TAMs from the BPA-exposed group showed a lower Fizz-1 relative expression mean (0.307; CI 0.09, 0.51) when compared to the intact (0.834; CI 0.52, 1.27) and vehicle groups (0.821; CI 0.47, 1.26) (Fig. 5b). Ym-1, on the other hand, was not expressed in any of the tumour samples.

In contrast to the tumour infiltrate, which usually shows Treg enrichment, the spleen of tumour-bearing mice appeared nearly devoid of these cells. Further, a major finding involved the virtual absence of NK cells from the tumour infiltrate and were drastically diminished in the periphery of all tumour-bearing mice, in contrast to healthy ones.

Intra-tumoral cytokine expression. As part of the tumour immunological microenvironment analysis, we determined the relative expression of pro-inflammatory (IL-1β, TNF-α, and IFN-γ), as well as regulatory (IL-10 and TGF-β) cytokines in tumour tissue. The cytokine milieu in all groups is clearly skewed towards a regulatory phenotype, as denoted by the predominance of TGF-β relative expression (Fig. 6e).

However, it drew our attention that, whilst having a greater regulatory T proportion, the tumour samples of the BPA-exposed group did not exhibit a greater expression of TGF-β, nor IL-10 (Fig. 6d,e). Moreover, both TNF-α and IFN-γ expression was decreased in the BPA-exposed group (Fig. 6b,c), suggesting that the overall tumour microenvironment in this group displays a lesser pro-inflammatory character.

ER-α expression. The cells of the immune system are modulated by sex steroids, mediated by receptors present in these cells. Given the oestrogenic character of the endocrine disruption caused by BPA, we analysed ERα expression in the main immune subpopulations at both local and systemic levels.

We observed a greater proportion of ERα-positive T lymphocytes in the tumour microenvironment of BPA-exposed mice, accompanied by an elevated expression level of the receptor (Fig. 7a).

The macrophage infiltrate of the BPA-exposed group also showed a larger percentage of ERα-positive cells; however, ERα expression in this group was significantly lower (Fig. 7b). This is a noteworthy finding, as it could account for the decreased Fizz-1 expression found in the same tissue, considering that oestrogen signalling promotes the expression of this protein[32].

At systemic level, we found that the T lymphocytes of tumour-bearing mice had lower ERα expression compared to their healthy counterparts (Fig. 7c). Though this phenomenon was observed in all groups, the deviation in ERα expression between healthy and tumour-bearing mice was greater in the Vehicle and BPA groups than in the Intact group. A similar behaviour was noted in splenic macrophages, where the proportion of ERα-positive cells was decreased in tumour-bearing mice of the Vehicle and BPA-exposed groups (Fig. 7d). As ERα expression remained constant in healthy mice regardless of the neonatal treatment, these findings suggest that neonatal stress, but not BPA exposure could play a role in the modulation of ERα expression in response to tumour development.

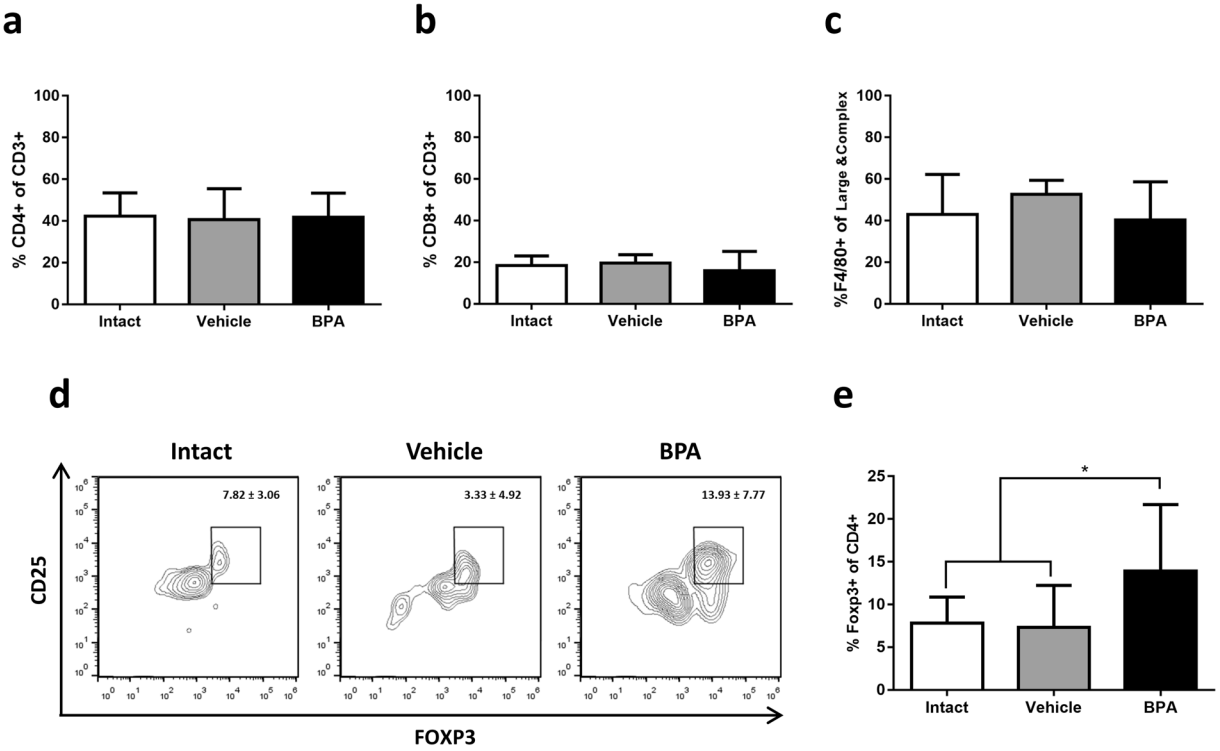

Figure 3. Analysis of tumour leucocytic infiltrate. Determination of immune subpopulations by flow cytometry, (**a**) T helper lymphocytes, (**b**) cytotoxic T lymphocytes, (**c**) macrophages and (**e**) Treg; $*p = 0.0335$ vs Intact, $p = 0.0247$ vs Vehicle. Data from 3 independent experiments are expressed as mean ± SD; n = 12. (**d**) Representative contour representations of cytometric analysis of Treg percentage; gate: $CD3^+$, $CD4^+$ from 10,000 lymphocytes collected.

The analysis of ERα expression in NK cells was only possible at systemic level, since this population was absent from the tumour microenvironment at this time point. Moreover, the spleens of tumour-bearing mice was depleted of NK cells. Nevertheless, the analysis on ERα expression in NK cells of healthy mice revealed that neonatal BPA-exposure not only increased the percentage of ERα positive cells, but also increased its expression level (Fig. 7e).

Discussion

Our experimental model involves the growth of a mammary adenocarcinoma tumour embedded within the mammary gland of a mouse[33], resulting in a hormone-sensitive system. Originally, the use of 4T1 cells was contemplated as a strategy excluding the use of oestradiol as a growth factor, given that this cell line does not express the oestrogen receptor ERα[34–36]; however, we detected the presence of this receptor in the 4T1 cell line. This posed the possibility that the observed changes in tumour growth could be attributed to either endocrine (*e.g.* higher serum oestradiol levels) or immunological factors, or both. Whilst it has been well documented that BPA administered in rodents exerts several effects on the endocrine system, particularly on the reproductive axis, such as early puberty onset, oestrous cycle alterations, and even altered development of the mammary gland[8,9,12,37], the majority of these effects occur after a prolonged perinatal or pre-pubertal exposure to higher doses[38].

Therefore, we evaluated the potential endocrine alterations produced by the tested exposure scheme, *i.e.* a single dose of 250 µg/kg bw at postnatal day 3. As a result, we did not observed any alterations regarding puberty onset, regularity of the oestrous cycle, or basal oestradiol levels in serum. The latter suggests that the observed effects, regarding tumour progression, are mainly due to immunological factors.

Remarkably, tumour growth was promoted as a result of a single neonatal BPA exposure. Previous studies have associated BPA with breast cancer risk, mainly as a consequence of mammary gland alterations and carcinogenesis susceptibility[14–17,39,40]. Nevertheless, our results show a new aspect whereby BPA can contribute to breast cancer development and progression by modifying the anti-tumoral immune response.

The analysis of the tumour leukocytic infiltrate showed that the BPA-exposed group had a larger proportion of Treg lymphocytes, in accordance with a greater tumour growth. It is known that Tregs express the immunomodulatory cytokines IL-10 and TGF-β while at the same time limiting the production of pro-inflammatory cytokines by other cells[41–43]. It appeared odd that we did not observe any changes in the expression of immunomodulatory cytokines in the BPA group; instead, this group showed a lower expression of the pro-inflammatory cytokines IFN-γ and TNF-α; therefore, the tumour microenvironment of this group was indeed modified in general terms. In any case, the expression level was assessed at mRNA level, which is limiting when considering the post-transcriptional regulations that may be affecting final protein synthesis. Regardless, the immunomodulatory

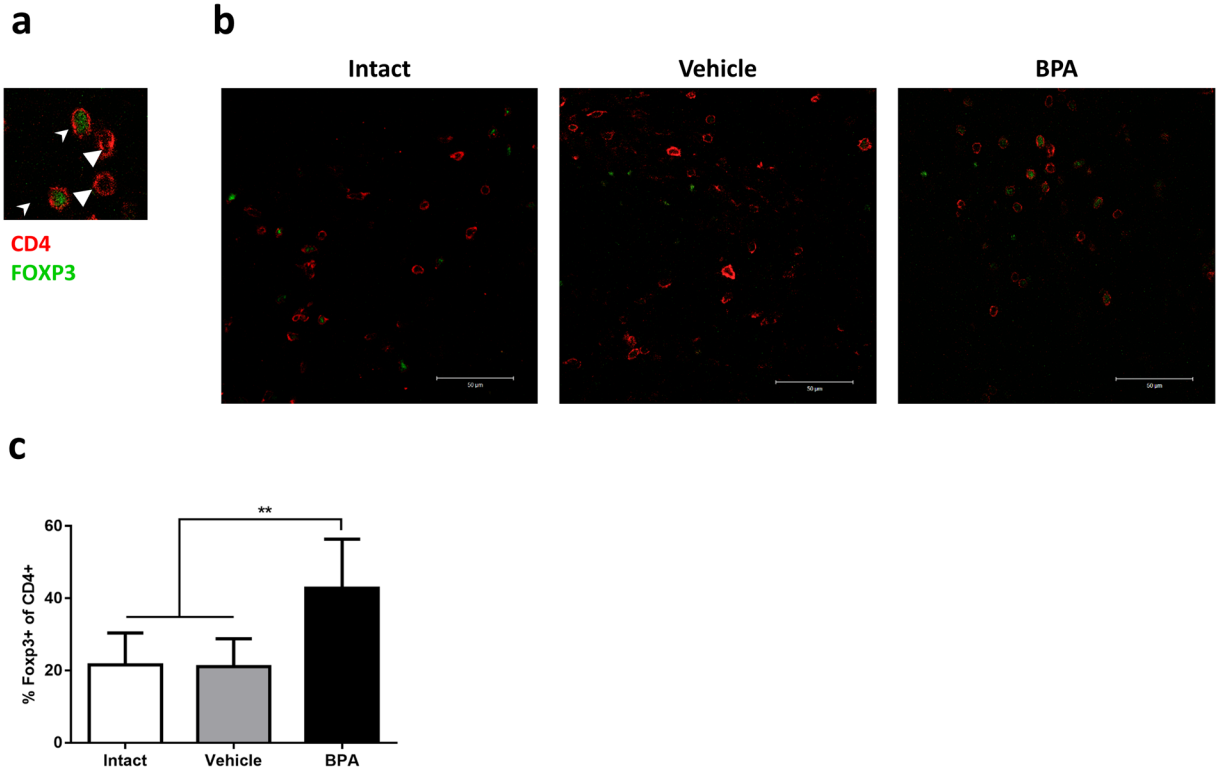

Figure 4. Evaluation of tumour Treg abundance by immunofluorescence. (**a**) Representative identification of T helper lymphocytes (white triangles △) and Treg (white arrowheads ⩓) by immunofluorescence staining of membranal CD4 (red) and nuclear Foxp3 (green); scale bar, 50 μm; original magnification, ×40. (**b**) Representative images of immunofluorescence staining of tumour samples. (**c**) Treg proportion expressed as the percentage of $Foxp3^+$ cells of $CD4^+$ cells; $^{**}p = 0.0031$ vs Intact, $p = 0.0070$ vs Vehicle; data from 2 independent experiments are expressed as mean ± SD; n = 8.

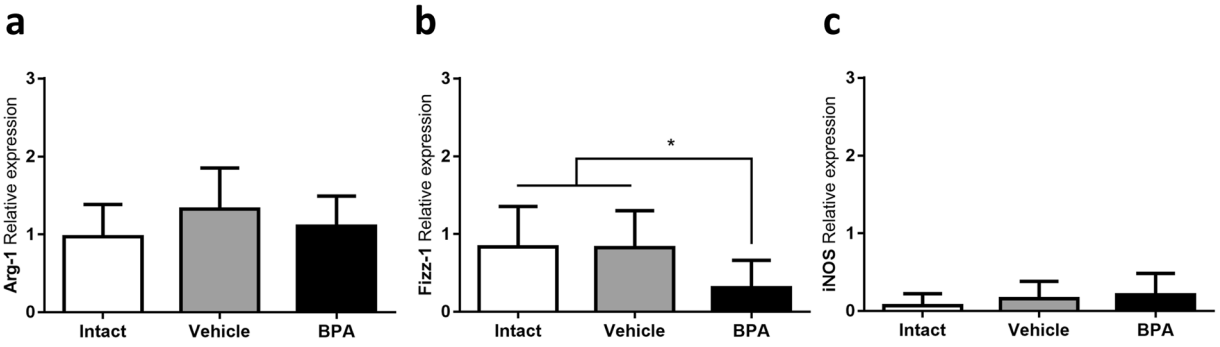

Figure 5. TAMs activation profile. Relative expression of alternative activation markers (**a**) Arginase and (**b**) Fizz-1 and classical activation marker (**c**) iNOs determined by RT-PCR. Expression relative to 18 S ribosomal subunit as constitutive expression reference. $^{*}p = 0.0326$ vs Intact, $p = 0.0476$ vs Vehicle; data from 2 independent experiments are expressed as mean ± SD; n = 10.

role of Treg lymphocytes is complex, as their effector mechanisms are not limited to the cytokine expression, and also mediate contact-dependent immunosuppression and IL-2 depletion.

Moreover, tumour microenvironment is not only defined by adaptive immunity cells; in fact, TAMs comprise a very important population. It is known that TAMs acquire a particular phenotype while in the tumour microenvironment, more similar to alternative (M2) than classical (M1) activation[44–46]. TAMS found in the analysed tumour samples did show a greater expression of arginase (M2 marker) compared to iNOS (M1 marker); however, it results interesting that the samples from BPA-exposed mice showed lower Fizz-1 expression. Although Fizz-1 is considered a M2 marker, the role of this protein is still not clear, and while it has been postulated that it can act as a modulator of type 2 inflammation in the lungs, there is no information concerning its role in the context of tumour microenvironment.

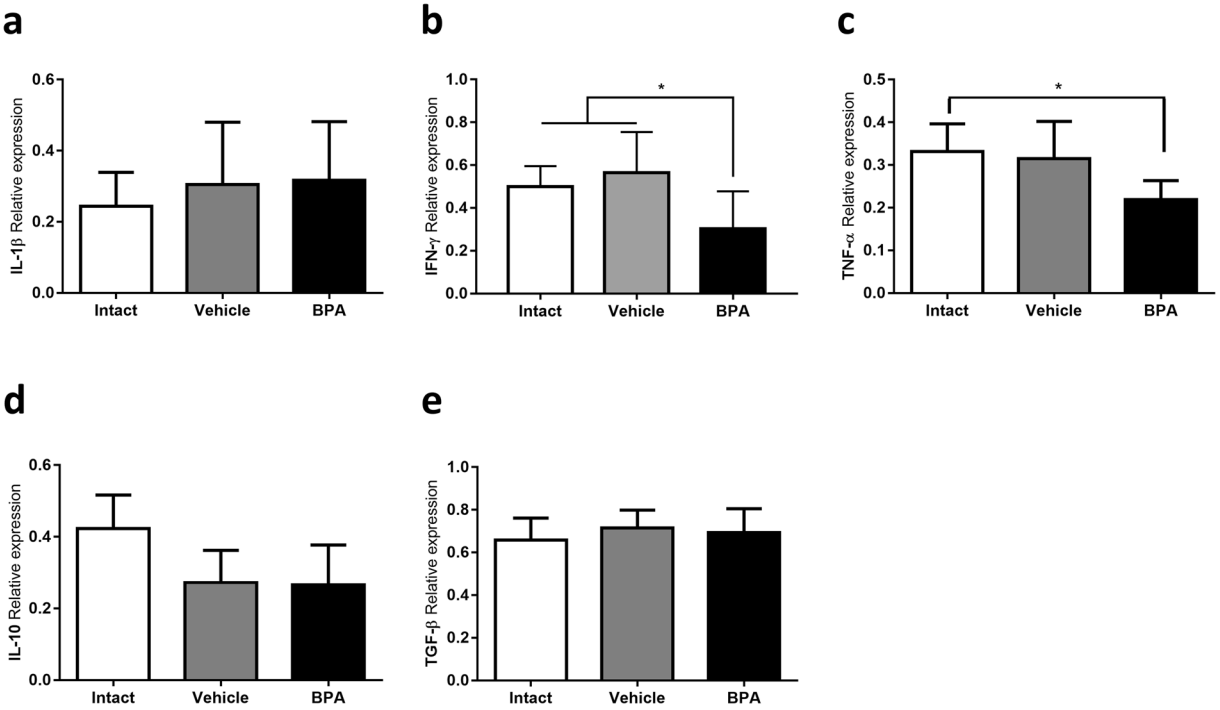

Figure 6. Intratumoural cytokine expression. Relative expression of proinflammatory (**a**) IL-1β, (**b**) IFN-γ, (**c**) TNF-α, and immunomodulatory cytokines (**d**) IL-10 and (**e**) TGF-β, determined by RT-PCR. Expression relative to 18 S ribosomal subunit as constitutive expression reference. In (**b**) $*p = 0.0216$ vs Intact, $p = 0.0034$ vs Vehicle; in (**c**) $*p = 0.0244$ vs Intact; data from 2 independent experiments are expressed as mean ± SD; n = 10.

One of the most relevant aspects of this study was the evaluation of the major oestrogen receptor in immune cells (ERα). As previously stated, sexual steroid hormones modulate the response of immune cells *via* its receptors; the effects that these hormones exert are diverse and greatly dependent on cell type, hormone concentration and combination, and even the expressed isoforms of the receptors[47–49]. Even in the context of an invariable hormone concentration, a change in the expression of a receptor may modify the sensibility of a cell to this hormone; in other words, it is not only a matter of how much hormone is present, but also how much the cell is sensing.

Considering that immune cells express a plethora of hormonal receptors, it is not possible to draw definitive conclusions based only on the evaluation of ERα. Nevertheless, it is possible to associate the changes in the expression of this receptor in immune cells to the changes observed in the modulation of these cells. For example, it has been reported that oestrogen promotes Treg differentiation *via* ERα[50–52], which could account in part for the increased Treg ratio in the tumour microenvironment, as T lymphocytes in this tissue showed a higher ERα expression level. Furthermore, oestrogen has also been shown to influence macrophage polarization, promoting an alternative activation during skin repair[32]. In this manner, the modulation of ERα in TAMs could also relate to the observed changes in their activation profile.

As for what caused the changes in ERα expression, it has been reported that BPA exposure during critical developmental stages is able to modify the epigenetic patterns, affecting the expression of oestrogen receptors in reproductive tissues and in the brain[20,53,54]. Considering the latter, it is a plausible possibility that immune cells are also subjected to epigenetic modulation due to developmental BPA exposure. However, such hypothesis needs to be confirmed.

Conclusion

The present work demonstrates that exposure to a single dose of BPA during the neonatal period induces changes in the immune system, leading to a differential anti-tumoral immune response during adulthood, causing greater tumour growth. This disparity is characterized by a greater intra-tumoral Treg proportion, decreased expression of pro-inflammatory cytokines, and a slightly different TAM activation profile. Because BPA exposure modified the expression pattern of ERα in immune cells, it is a plausible mechanism underlying the altered immune response caused by BPA exposure.

Methods

Ethics statement. Animal care and experimental practice were conducted at the Unidad de Modelos Biológicos (UMB) in the Instituto de Investigaciones Biomédicas (IIB), Universidad Nacional Autónoma de México. All experimental procedures in the animals were approved by the Institutional Care and Animal Use Committee (CICUAL), permit number 155, adhering to Mexican regulation (NOM-062-ZOO-1999), and in accordance with the recommendations from the National Institute of Health (NIH) of the United States of

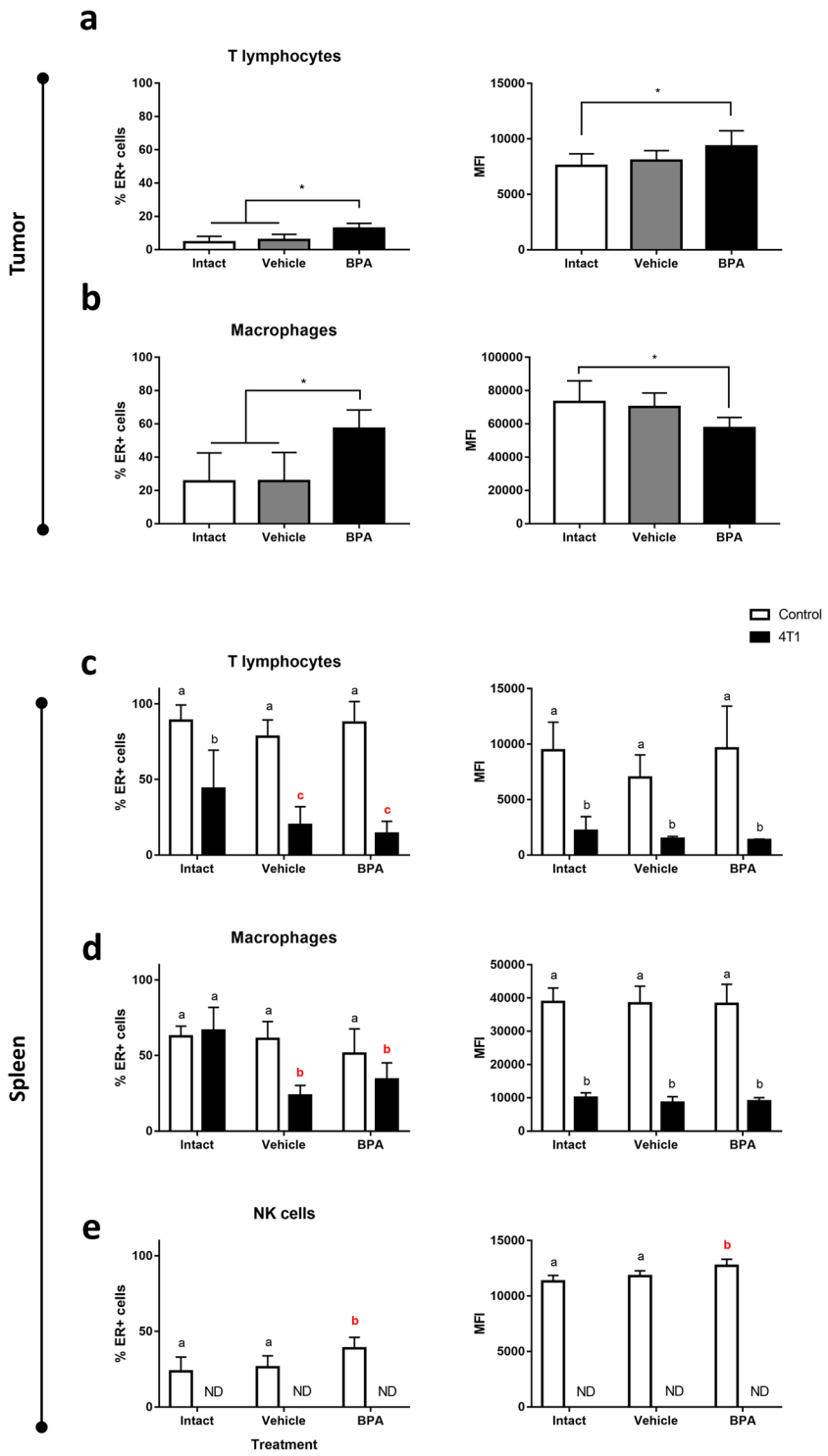

Figure 7. Flow cytometry evaluation of ERα expression in immune cells. Percentage of ERα positive cells (left panel) and ERα expression level (right panel) of immune cells in the tumour microenvironment (**a**,**b**) and in the spleen (**c**–**e**). (**a**,**c**) T lymphocytes, (**b**,**d**) macrophages, (**e**) NK cells. Data from 2 independent experiments are expressed as mean ± SD; n = 10. *$p < 0.05$ compared with unexposed controls. In (**c**–**e**) white bars, control = no tumour induction; black bars, 4T1 = tumour induction; letters above each column indicate statistical differences among groups: a, no significant difference; b, $p < 0.05$ compared to a; c, $p < 0.05$ compared to b; ND, no data available since this subpopulation was absent from the tissue.

America (Guide for the Care and Use of Laboratory Animals). Euthanasia of experimental animals was performed humanely by cervical dislocation after anaesthesia with 5% sevofluorane (Abbot, México).

Animals. Mice of the syngeneic strain BALB/c AnN (H2-d) were purchased from Harlan México (Facultad de Química, UNAM, México). The animals were housed at UMB with controlled temperature (22 °C) and 12-hrs light-dark cycles, with water and Purina LabDiet 5015 (Purina, St. Louis MO) chow *ad libitum*. After neonatal treatment, only female mice were used for experimentation.

Neonatal BPA exposure. To resemble the human final gestational stage and aiming at the murine critical T lymphocytes developmental window, mice were exposed at PND3.

Briefly, 72 hours after birth female pups were identified by ano-genital distance. Only female pups received treatment, though whole litters were assigned to experimental groups to avoid pup reallocation stress. The Intact group received no neonatal treatment. The vehicle group received a dorsal subcutaneous injection of 20 µl corn oil vehicle (Sigma, St. Louis MO). The BPA group received 250 µg/kg bw of BPA. Given that neonate rodents have minimal glucuronidation activity, which is the major metabolic mechanism for BPA clearance[55, 56], this dose approximates to a brief, 5 day exposure to the FDA reference dose of 50 µg/kg bw/day, but performed in a single administration, thus avoiding excessive manipulation stress.

Though the main exposure route is commonly oral, subcutaneous injection was selected instead as no difference between oral and subcutaneous routes are observed in neonate mice in this case[57].

Pups were weaned at 21 days of age and placed in standard cages, 5 mice per cage.

Assessment of endocrine parameters. Vaginal opening. From 25 days old forth, the vaginal opening was examined by holding the mice in a dorsal restraint and using a light extension of the peri-vaginal skin.

Estrous cycle. At 8 weeks old, the oestrous cycle was assessed using a vaginal smear wash of 50 µl saline solution (PiSA, Guadalajara México), followed by Giemsa stain and light microscope observation.

Serum Oestradiol. Serum samples obtained at sacrifice, corresponding to the diestrus phase, were used to determine oestradiol levels, using EIA DetectX® Serum 17βOestradiol kit (Arbor Assays, Ann Arbor MI), according to manufacturer's protocol.

Cell culture. The 4T1 cell line (ATCC® CRL-2539) was kindly donated by Dr. Pedro Ostoa-Saloma and cultivated in RMPI 1640 medium (Sigma, St. Louis MO) supplemented with 10% FBS (ByProductos, Guadalajara México). Subculture was performed at 70–80% confluency. After a second subculture, the cells were harvested and resuspended in 0.9% saline to a concentration of 250,000 cells/ml for inoculation.

Mammary tumour induction. Upon sexual maturity (8 weeks old), mice of every exposure group were randomized into secondary experimental groups, *i.e.* Control (without tumour induction) and 4T1 (tumour induction) groups. Mice assigned to 4T1 groups were treated as follows:

Mice were anesthetized by inhalation of a mixture of air and 5% sevofluorane. After low abdomen asepsis, 4th nipple was located and 10^4 4T1 cells were introduced by a single injection into the mammary fat pad. Tumour growth was monitored for 25 days.

Flow cytometry. The spleen was mechanically disaggregated using a 50 µm nylon mesh and washed with PBS. Spleen erythrocytes were lysed with ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) for 10 minutes and washed with PBS. Tumour samples were finely cut and incubated 20 minutes in digestion medium (RPMI 1640, 10 U/ml DNase (Roche, Mannheim Germany), 0.5 mg/ml type IV Collagenase (Sigma, St. Louis MO)). Digestion was stopped by adding 50 µl FBS and mesh disaggregation was performed, followed by PBS wash. Cells from all tissues were resuspended in FACS buffer (PBS, 2% FBS, 0.02% NaN_3).

Approximately 1×10^6 cells were incubated with anti-CD16/CD23 (TruStain®, BioLegend, San Diego CA) for 30 minutes at 4 °C, washed and stained. For the characterization of cellular subpopulations, the following antibodies were used: APCCy7-conjugated anti- CD3ε (145-2C11), PE-conjugated anti-CD4 (GK1.5), AlexaFluor®647-conjugated anti-Foxp3 (150D), PerCP-conjugated anti-CD8 (53–6.7), AlexaFluor®647-conjugated anti-F4/80 (BM8), PE-conjugated anti-NKp46 (29A1.4) (all from BioLegend, San Diego CA), and VioletFluor®450-conjugated anti-CD25 (PC61.5) (Tonbo biosciences, San Diego CA). For intra-nuclear staining, Foxp3/Transcription Factor Staining Buffer kit (Tonbo biosciences, San Diego CA) was used according to manufacturer's protocol. For oestrogen receptor alpha detection, a rabbit polyclonal anti-ERα (H-184) (Santa Cruz bt., Dallas TX) was used, followed by DyLight®488-conjugated Donkey anti-Rabbit IgG (BioLegend, San Diego CA). Using an Attune cytometer (Life Technologies) with blue and red laser, the obtained data was analysed with the FlowJo software (Treestar Inc.).

Immunofluorescence. Tumour tissue samples were fixed in 4% paraformaldehyde (in PBS pH 7.1) for 20 minutes, cryoprotected in 30% sucrose (in PBS, pH 7.1) overnight, embedded in O.C.T. compound (Sakura Finetek, Torrance CA) and frozen at −70 °C. 10 µm thick sections were washed with PBS, permeabilised with 0.1% Triton X-100 (Sigma, St. Louis MO) in PBS for 10 min and blocked with 1% Bovine Serum Albumin (Sigma, St. Louis MO) for 2 hours. The sections were then incubated overnight at 4 °C with PE-conjugated anti-CD4 (GK1.5) and AlexaFluor®647-conjugated anti-Foxp3 (150D) (BioLegend, San Diego CA). After thoroughly washing with PBS, the sections were mounted in fluorescence mounting medium Fluoroshield® (Sigma, St. Louis MO) and stored at 4 °C until examination with confocal microscope (LSM5 Pascal, Carl Zeiss).

Oligonucleotide	Sequence	MT (°C)	Product (bp)
IL-1β Sense	TCATGGGATGATGATGATAACCTGCT	62	502
IL-1β Antisense	CCCATACTTTAGGAAGACACGGATT		
IL-10 Sense	AACTGGTAGAAGTGATGCCCCAGGCA	63	237
IL-10 Antisense	CTATGCAGTTGATGAAGATGTCAAA		
TNF-α Sense	GGCAGGTCTACTTTGGAGTCATTGC	63	300
TNF-α Antisense	ACATTCGAGGCTCCAGTGAATTCGG		
IFN-γ Sense	AGCGGCTGACTGAACTCAGATTGTAG	60	247
IFN-γ Antisense	GTCACAGTTTTCAGCTGTATAGGG		
TGF-β Sense	CTTCAGCTCCACAGAGAAGAACTGA	61	298
TGF-β Antisense	CACAATCATGTTGGACAACTGCTCC		
18S Sense	CGCGGTTCTATTTTGTTGGT	60	219
18S Antisense	AGTCGGCATCGTTTATGGTC		
Arg-1 Sense	CTTGCGAGACGTAGACCCTG	64	387
Arg-1 Antisense	TGAGTTCCGAAGCAAGCCAA		
Fizz-1 Sense	GGTCCCAGTGCATATGGATGAGAC	58	296
Fizz-1 Antisense	CACCTCTTCACTCGAGGGACAGTT		
YM-1 Sense	TCACAGGTCTGGCAATTCTTCTG	60	437
YM-1 Antisense	TTTGTCCTTAGGAGGGCTTCCTC		
iNOS Sense	CAGCTCCACAAGCTGGCTCG	63	657
iNOS Antisense	CAGGATGTCCTGAACGTAGACCTT		

Table 1. Oligonucleotide sequences used for RT-PCR.

RT-PCR. Tumour tissue samples were frozen in TRIzol® reagent (Ambion, Carlsbad CA) immediately after recollection. Total RNA was extracted with same reagent, following manufacturer's protocol. Briefly, the tissue was disrupted in TRIzol® reagent (1 ml/0.1 g tissue) and 0.2 ml of chloroform was added per each ml of reagent. After centrifugation at 13,000 rpm for 15 minutes, the aqueous phase was recovered. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and dissolved in RNAse-free water. RNA concentration was determined by absorbance at 260 nm, and its integrity was verified following electrophoresis on 1.0% agarose gel.

Total RNA samples were immediately reverse-transcribed, using M-MLV Reverse Transcriptase (Promega, Madison WI) and dT12–18 primers (Invitrogen, USA). Then, cDNA was specifically amplified by semi-quantitative PCR, using TaqDNA polymerase (Biotecnologías Universitarias, UNAM. México) and *Mus musculus*-specific primers (see Table 1). The relative expression of each amplified gene was obtained by densitometric analysis, using the 18S-ribosomal RNA amplicon as a constitutive control.

Statistical analysis. The general experimental design considers 2 independent variables: neonatal exposure (Intact, Vehicle of BPA) and mammary tumour induction (Control or 4T1). The data regarding tumour development and tumour microenvironment only considers the exposure variable, as all animals belong to 4T1 group. Data from 2-3 independent experiments are charted as mean ± standard deviation and analysed with Prism 6® software (GraphPad Software Inc.). Data distribution normality was assessed *via* Shapiro-Wilk test. Thereafter, a one-way ANOVA ($\alpha = 0.05$) was performed, followed by a Tukey *post-hoc* test. Differences were considered significant when $p < 0.05$, with the actual p value being stated in each figure legend. The data regarding oestrogen receptor expression considers both independent variables and therefore, a two-way ANOVA ($\alpha = 0.05$) was performed, followed by a Holm-Šidák *post-hoc* test, with the same significant difference criterion.

Data availability. The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

References

1. Diamanti-Kandarakis, E. *et al*. Endocrine-Disrupting Chemicals. *Endocr. Rev.* **30**, 293–342 (2009).
2. Wetherill, Y. B. *et al*. *In vitro* molecular mechanisms of bisphenol A action. *Reprod. Toxicol.* **24**, 178–198 (2007).
3. Matthews, J. B., Twomey, K. & Zacharewski, T. R. *In Vitro* and *in Vivo* Interactions of Bisphenol A and Its Metabolite, Bisphenol A Glucuronide, with Estrogen Receptors a and b. *Chem. Res. Toxicol.* **14** (2001).
4. Le, H. H., Carlson, E. M., Chua, J. P. & Belcher, S. M. Bisphenol A is released from polycarbonate drinking bottles and mimics the neurotoxic actions of estrogen in developing cerebellar neurons. *Toxicol. Lett.* **176**, 149–156 (2008).
5. Goodson, A., Robin, H., Summerfield, W. & Cooper, I. Migration of Bisphenol A from can coatings — effects of damage, storage conditions and heating. *Food Addit. Contam.* **21**, 1015–1026 (2004).
6. Fleisch, A. F., Sheffield, P. E., Chinn, C., Edelstein, B. L. & Philip, J. Bisphenol A and Related Compounds in Dental Materials. *Pediatrics* **126**, 760–768 (2010).
7. Honma, S. *et al*. Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. *Reprod. Toxicol. Elmsford NY* **16**, 117–122 (2002).
8. Nah, W. H., Park, M. J. & Gye, M. C. Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and estrous cycle in female mice. *Clin. Exp. Reprod. Med.* **38**, 75–81 (2011).

9. Rasier, G. & Toppari, J. Parent, a-S. & Bourguignon, J.-P. Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: a review of rodent and human data. *Mol. Cell. Endocrinol.* **254–255**, 187–201 (2006).
10. Timms, B. G. *et al*. Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. *Proc. Natl. Acad. Sci. USA*. **102**, 7014–7019 (2005).
11. Flick, B., Mayr, E., Talsness, C., Paul, M. & Chahoud, I. In Utero Exposure to Low Doses of Bisphenol A Lead to Long-term Deleterious Effects in the Vagina 1. **4**, 98–102 (2002).
12. Dhimolea, E. *et al*. Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the propensity to neoplastic development. *PLoS One* **9**, e99800 (2014).
13. Fenton, S. E. Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences. *Endocrinology* **147**, S18–24 (2006).
14. Yang, L. *et al*. Effect of low dose bisphenol A on the early differentiation of human embryonic stem cells into mammary epithelial cells. *Toxicol. Lett.* **218**, 187–93 (2013).
15. Tharp, A. P. *et al*. Bisphenol A alters the development of the rhesus monkey mammary gland. *Proc. Natl. Acad. Sci. USA*. **109**, 8190–5 (2012).
16. Vandenberg, L. N. *et al*. Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. *Endocrinology* **148**, 116–27 (2007).
17. Muñoz-de-Toro, M. *et al*. Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. *Endocrinology* **146**, 4138–47 (2005).
18. Rubin, B. S. *et al*. Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. *Endocrinology* **147**, 3681–91 (2006).
19. Itoh, K., Yaoi, T. & Fushiki, S. Bisphenol A, an endocrine-disrupting chemical, and brain development. 447–457, doi:10.1111/j.1440-1789.2011.01287.x (2012).
20. Kundakovic, M. *et al*. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proc. Natl. Acad. Sci. USA*. **110**, 9956–61 (2013).
21. Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y. & Taketani, Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum. Reprod.* **17**, 2839–41 (2002).
22. Vandenberg, L. N. *et al*. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environ. Health Perspect.* **118**, 1055–70 (2010).
23. Landreth, K. S. Critical windows in development of the rodent immune system. *Hum. Exp. Toxicol.* **21**, 493–498 (2002).
24. Sakaguchi, S., Takahashi, T. & Nishizuka, Y. Study on cellular events in postthymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J. Exp. Med.* **156** (1982).
25. Bonomo, A., Kehn, P. J. & Shevach, E. M. Post-thymectomy autoimmunity: abnormal T-cell homeostasis. *Immunol. Today* **16**, 61–67 (1995).
26. Classen, J. B. Cyclosporine Induced Autoimmunity in Newborns Prevented by Early Immunization. *Autoimmunity* **27**, 135–139 (1998).
27. WHO. Cancer Fact Sheet. *Cancer Fact Sheet* (2017). Available at: http://www.who.int/mediacentre/factsheets/fs297/en/. (Accessed: 25th February 2017).
28. Dunn, G. P. *et al*. The immunobiology of Cancer Immunosurveillance and Immunoediting. *Immunity* **21**, 137–148 (2004).
29. Smyth, M. J., Dunn, G. P. & Schreiber, R. D. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv. Immunol.* **90**, 1–50 (2006).
30. Da Silva, J. A. Sex hormones and glucocorticoids: interactions with the immune system. *Annals of the New York Academy of Sciences* **876**, 102–17–8 (1999).
31. Muñoz-Cruz, S., Togno-Pierce, C. & Morales-Montor, J. Non-Reproductive Effects of Sex Steroids: Their Immunoregulatory Role. Or The Regulatory Role of Sex Steroids on Innate and Adaptive Immunity. *Curr. Top. Med. Chem.* **11** (2011).
32. Campbell, L. *et al*. Estrogen Receptor-Alpha Promotes Alternative Macrophage Activation during Cutaneous Repair. *J. Invest. Dermatol.* **134**, 2447–2457 (2014).
33. Pulaski, B. A. & Ostrand-Rosenberg, S. Mouse 4T1 breast tumor model. *Curr. Protoc. Immunol.* Chapter 20, Unit 20.2 (2001).
34. Zhang, Y. *et al*. A novel role of hematopoietic CCL5 in promoting triple-negative mammary tumor progression by regulating generation of myeloid-derived suppressor cells. *Cell Res.* **23**, 394–408 (2013).
35. Singh, M. *et al*. Curcumin improves the therapeutic efficacy of Listeria ^{at} -Mage-b vaccine in correlation with improved T-cell responses in blood of a triple-negative breast cancer model 4T1. *Cancer Med.* **2**, 571–582 (2013).
36. Yang, X. *et al*. Estradiol increases ER-negative breast cancer metastasis in an experimental model. *Clin. Exp. Metastasis* **30**, 711–21 (2013).
37. Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G. & Vom Saal, F. S. Exposure to bisphenol A advances puberty. *Nature* **401**, 763–764 (1999).
38. Richter, C. A. *et al*. *In Vivo* Effects of Bisphenol A in Laboratory Rodent Studies. *Reprod. Toxicol.* **24**, 199–224 (2008).
39. Murray, T. J., Maffini, M. V., Ucci, A. A., Sonnenschein, C. & Soto, A. M. Induction of mammary gland ductal hyperplasias and carcinoma *in situ* following fetal bisphenol A exposure. *Reprod. Toxicol.* **23**, 383–90 (2007).
40. Durando, M. *et al*. Prenatal Bisphenol A Exposure Induces Preneoplastic Lesions in the Mammary Gland in Wistar Rats. *Environ. Health Perspect.* **115**, 80–86 (2006).
41. Wang, H. Y. & Wang, R.-F. Regulatory T cells and cancer. *Curr. Opin. Immunol.* **19**, 217–23 (2007).
42. Shevach, E. M. *et al*. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol. Rev.* **212**, 60–73 (2006).
43. Ghiringhelli, F., Ménard, C., Martin, F. & Zitvogel, L. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunol. Rev.* **214**, 229–38 (2006).
44. Murray, P. J. & Wynn, T. A. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* **11**, 723–37 (2011).
45. Qian, B.-Z. & Pollard, J. W. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141**, 39–51 (2010).
46. Mantovani, A., Sica, A., Allavena, P., Garlanda, C. & Locati, M. Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum. Immunol.* **70**, 325–30 (2009).
47. Nilsson, S., Ma, S., Treuter, E., Tujague, M. & Thomsen, J. Mechanisms of Estrogen Action **81** (2001).
48. Diel, P. Tissue-specific estrogenic response and molecular mechanisms. *Toxicol. Lett.* **127**, 217–24 (2002).
49. Filardo, E. J. & Thomas, P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology* **153**, 2953–62 (2012).
50. Polanczyk, M. J. *et al*. Cutting edge: estrogen drives expansion of the CD4+ CD25+ regulatory T cell compartment. *J. Immunol.* **173**, 2227–2230 (2004).
51. Ohkura, N., Hamaguchi, M. & Sakaguchi, S. FOXP3+ regulatory T cells: control of FOXP3 expression by pharmacological agents. *Trends Pharmacol. Sci.* **32**, 158–66 (2011).
52. Tai, P. *et al*. Induction of regulatory T cells by physiological level estrogen. *J. Cell. Physiol.* **214**, 456–464 (2008).
53. Doshi, T., Mehta, S. S., Dighe, V., Balasinor, N. & Vanage, G. Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. *Toxicology* **289**, 74–82 (2011).
54. Santangeli, S. *et al*. BPA-Induced Deregulation Of Epigenetic Patterns: Effects On Female Zebrafish Reproduction. *Sci. Rep.* **6**, 21982 (2016).

55. Matsumoto, J., Yokota, H. & Yuasa, A. Developmental increases in rat hepatic microsomal UDP-glucuronosyltransferase activities toward xenoestrogens and decreases during pregnancy. *Environ. Health Perspect.* **110**, 193–6 (2002).
56. Doerge, D. R., Twaddle, N. C., Woodling, K. A. & Fisher, J. W. Pharmacokinetics of bisphenol A in neonatal and adult rhesus monkeys. *Toxicol. Appl. Pharmacol.* **248**, 1–11 (2010).
57. Taylor, J. A., Welshons, W. V. & Vom Saal, F. S. No effect of route of exposure (oral; subcutaneous injection) on plasma bisphenol A throughout 24h after administration in neonatal female mice. *Reprod. Toxicol.* **25**, 169–76 (2008).

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Author Contributions

M.I.P.A. wrote the main manuscript text and was responsible for the experimental procedures. V.H.R.A. and N.Y.P.S. contributed to the experimental work. K.E.N.C. and J.M.M. supervised the work. All authors reviewed the manuscript.

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Anexo II

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Review Article

The Role of Chemokines in Breast Cancer Pathology and Its Possible Use as Therapeutic Targets

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Chemokines are small proteins that primarily regulate the traffic of leukocytes under homeostatic conditions and during specific immune responses. The chemokine-chemokine receptor system comprises almost 50 chemokines and approximately 20 chemokine receptors; thus, there is no unique ligand for each receptor and the binding of different chemokines to the same receptor might have disparate effects. Complicating the system further, these effects depend on the cellular milieu. In cancer, although chemokines are associated primarily with the generation of a protumoral microenvironment and organ-directed metastasis, they also mediate other phenomena related to disease progression, such as angiogenesis and even chemoresistance. Therefore, the chemokine system is becoming a target in cancer therapeutics. We review the emerging data and correlations between chemokines/chemokine receptors and breast cancer, their implications in cancer progression, and possible therapeutic strategies that exploit the chemokine system.

1. Introduction

Chemokines are small proteins that primarily regulate the trafficking of leukocytes under homeostatic conditions and during specific immune responses. They share a secondary structure, and based on their amino acid composition—specifically, the presence of a conserved tetra-cysteine motif—they are grouped into 4 families: C, CC, CXC, and CX3C [1, 2]. Chemokines guide the migration and adhesion of leukocytes and influence other cellular functions, such as proliferation, maturation, angiogenesis, and malignant transformation [1, 2]. These effects are mediated by binding to G-protein-coupled receptors (GPCRs) with 7 transmembrane domains [2].

The chemokine system comprises almost 50 chemokines and approximately 20 chemokine receptors [2, 3]. Upon the binding of different chemokines to the same receptor, there can be a variety of biological effects. Complicating the system further, the effects also depend on the cellular microenvironment.

Breast cancer has gained particular relevance in recent years due to the high incidence in both developed and less developed regions [4]. Breast cancer is basically defined by the presence of a malignant tumor that originates from breast tissue, either from lobes, ducts, or stroma. The tumor cells proliferate and are able to invade surrounding tissues, lymph nodes and distant organs. According to the size of the primary tumor, the involvement of lymph nodes, and the presence of distant metastasis, the stage of breast cancer can be determined, ranging from stage 0 to stage IV [5].

The intervention of the immune system in cancer does not begin with the fighting and effort to restrain an established tumoral mass but with the detection of transformed cells since they began proliferating. In the late 50's, Burnet proposed the *immunosurveillance* theory [6], which proposes that the immune system cells are capable of detecting

transformed cells, attacking them and eliciting an specific (adaptive) response to eventually succeed and eliminate them all or fail, leading then to the formation of a tumoral mass and cancer onset.

Nowadays, it is known that this process is more complex than previously thought and consists not only of that simple event series, but can comprise alternative processes, such as *immunoediting* and even *immunosubversion* [7, 8]. The previous could be depicted by the paradoxical fact that a greater infiltration of immune cells in breast cancer neoplasia has been correlated with a worse disease prognostic, and how this can also be explained by the polarization phenomena that immune cells experience in the tumoral microenvironment, which induces the acquisition of a protumoral phenotype.

Chemokines and chemokine receptors play a key role along these processes, since they not only comprise the main regulatory system leading leukocyte infiltration in primary tumors, but also intervene in cancer cells proliferation and in metastasis guidance.

Nearly every tissue expresses chemokines and chemokine receptors. Normal breast expresses a set of chemokines at generally low levels [9]. We review the emerging data and correlations between chemokines and breast cancer, from their implications in cancer progression to therapeutic strategies that exploit the chemokine system.

2. Breast Cancer Cells Proliferation and Tumor Growth: Is There a Function for Chemokines?

Chemokines not only are associated with the establishment of a protumoral microenvironment and organ-directed metastasis, but also mediate disease progression, favoring the growth and proliferation of tumor cells. Several chemokines have been described as participating in these processes.

One of those chemokines implicated in breast cancer progression is CCL2 (formerly known as MCP-1), which is a potent chemotactic factor that regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells, signaling through CCR2 and CCR4 [10]. CCL2 is expressed at high levels in both tumor and tumor microenvironment cells, exerting its protumoral effects indirectly by promoting angiogenesis and enriching leukocyte infiltration [11, 12], primarily with tumor-associated macrophages (TAMs), which produce immunomodulatory factors that promote angiogenesis and tumor growth.

Moreover, CCL2 mediates development of the cancer stem cell (CSC) phenotype. Cancer-associated fibroblasts (CAFs) and fibroblasts that are activated by coculture with cancer cells secrete high levels of CCL2, which affects the sphere-forming phenotype (stem cell-specific) of breast cancer cells and CSC self-renewal [13]. The promotion or CSC phenotype is of great relevance in cancer biology, given that this population of self-renewing, chemo- and radioresistant cells is thought to maintain the tumor heterogeneity, as well as giving rise to metastasis.

Another member of the CC family of chemokines that has been related to breast cancer progression is CCL20. This chemokine primarily targets lymphocytes and dendritic cells, although it also attracts neutrophils weakly, upon binding to receptor CCR6. Recently, Marsigliante and colleagues [14] correlated high CCL20 concentrations with extensive cellular proliferation, mediated by increased cyclin E (which is required for the transition from G1 to S phase) and decreased p27 (an inhibitor of cyclin D).

Cell cycle regulation is clearly a key element in cancer progression and recently CXCL8, a member of the CXC family, has been identified as a promoter of cell cycle progression. Shao and colleagues demonstrated that silencing CXCL8 using siRNA in the MDA-MB-231 breast cancer cell line resulted in the upregulation of p27, downregulation of cyclin D1, and thus a delay in the progression from G1 to S phase [15]. Besides cell cycle regulation, CXCL8 has been implicated in CSC phenotype. It has been reported that breast cancer stem cells express CXCR1, which upon binding of CXCL8 increase their activity (measured as sphere-formation) and self-renewal [16].

Although traditionally implicated in organ-directed metastasis, CXCR4 is a chemokine receptor that has been linked to cancer progression. Its constitutive activation in MCF-7 breast cancer cells enhances tumor growth and metastasis, which can be reversed by its inhibition [17].

In contrast, the chemokine CXCL14, which is abundantly expressed in normal tissue but downregulated in breast cancer tissue and cell lines, negatively regulates the growth and metastasis of breast cancer as its expression is positively associated with patient survival and a lower incidence of metastasis. Overexpression of CXCL14 was recently reported to inhibit cell proliferation *in vitro* and decrease xenograft tumor growth *in vivo* [18].

3. Chemokines and Tumor Microenvironment

Cancer cells are not the only decisive factor in the course of the disease—there are other factors, both systemic and in the tumor microenvironment, that can limit or promote the growth of cancer cells and their mobility and dissemination to other organs.

When talking about the progression of cancer, one of the key elements of the tumor microenvironment is the myeloid cell population, particularly macrophages. On recruitment to the tumor microenvironment, macrophages are influenced by the cytokine milieu and local growth factors, resulting in the acquisition of a protumoral phenotype. The resulting TAMs produce angiogenic and immunomodulatory factors (e.g., IL-10 and TGF-β) which induce regulatory T lymphocytes (T_{reg}) and facilitate remodeling of the extracellular matrix, promoting cancer cell motility. Because a primary function of chemokines is to attract and direct leukocytes (Figure 1), their significance is evident, regarding leukocyte infiltration into the tumor microenvironment.

CCL2 and CCL5 (RANTES) chemokines have been extensively studied in breast cancer. As mentioned before, CCL2 is a potent chemoattractant of monocytes (Figure 1); in addition to monocytes, CCL5 recruits T helper type 2 lymphocytes (Th2) and eosinophils, signaling through

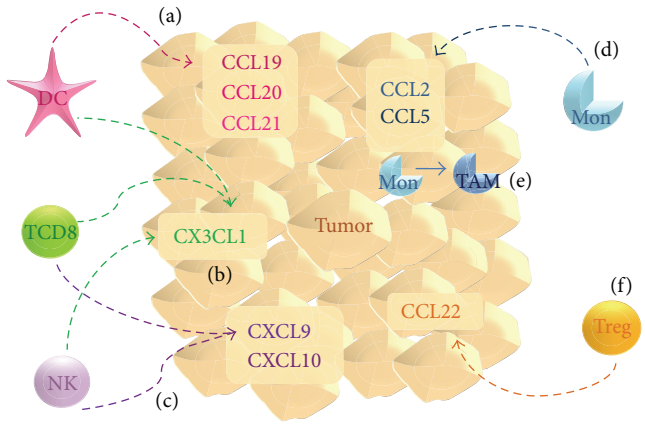

Figure 1: Chemokines influx the tumor microenvironment. (a) CCL19, CCL20, and CCL21 act as chemoattractants for dendritic cells (DC); (b) CX3CL1 has been related to infiltration of DCs as well as activated (cytotoxic) CD8+ T lymphocytes and NK cells. These last two populations are also chemoattracted by (c) CXCL9 and CXCL10. DCs, T CD8+ lymphocytes, and NK cells are thought to contribute to antitumoral immune response. (d) CCL2 and CCL5 are both chemoattractants for monocytes (Mon), which (e) within tumor microenvironment acquire a TAM phenotype. (f) CCL22 expression correlates with T_{reg} infiltration, which together with TAMs promotes tumor survival and progression.

the receptors CCR1, CCR3, and CCR5 [2, 11]. These chemokines have similar expression patterns—they are detected in cancer cells in primary tumors, tumor-infiltrated lymph nodes, distant metastases, and cells that are adjacent to the tumor (e.g., TAMs and fibroblasts) but are expressed at low levels in healthy breast tissue [11, 12].

An increase in the CCL2 expression levels correlate with the extent of TAM infiltration in primary tumors and in animal xenograft models, a causal relationship based on findings where blocking CCL2 with neutralizing antibodies decreases macrophage infiltration, tumor growth, and angiogenesis (associated to some extent with the presence of TAMs) in a mouse model of breast cancer [19, 20]. Although CCL5 is also linked to macrophage infiltration in animal models of breast cancer [21, 22], in human breast xenografts, CCL5 expression correlates negatively with macrophage recruitment [19], implying the participation of other chemokines.

In addition to macrophages, the leukocyte infiltrate in the tumoral niche includes T lymphocytes, dendritic cells (DCs), NK cells, and other granulocytes, which also influence the fate of cancer cells at the cellular level and by modifying the tumoral microenvironment with cytokines and chemokines. The chemokines CCL19, CCL20, and CCL21 regulate the traffic of DCs (Figure 1), and their overexpression in experimental tumor systems has antitumoral effects [12]. Although the function of DCs in cancer immunology is still under investigation, the effects of the overexpression of these chemokines correlate with increased DC infiltration.

T lymphocytes and NK cells are important populations in tumor immunology—activated lymphocytes orchestrate immune response against cancer cells, and NK cells are central innate effectors that recognize and have cytotoxic effects on stressed and transformed cells. In this context, CXC chemokines CXCL9 (also known as Mig) and CXCL10 (IP-10) control the migration of activated T cells and NK cells [23, 24], which can enhance the antitumoral response (Figure 1). Moreover, these chemokines belong to the antiangiogenic ELR(−) CXC subfamily (further discussed later) [25, 26]. In addition, CXCL12 (SDF-1) was recently shown to promote $CD8^+$ cytotoxic T lymphocyte activity when overexpressed in a syngeneic model of breast carcinoma [27].

CX3CL1 (also known as Fractalkine) is the only known member of the CX3C chemokine family and signals through CX3CR1. This receptor is expressed on monocytes, NK cells, and T lymphocytes, to mediate several functions, including migration, adhesion, and proliferation [28]. As discussed, T lymphocytes and NK cells are key populations in antitumoral immunity—a response in which CX3CL1 is thus expected to be involved. Park and colleagues [29] described a positive correlation between CX3CL1 expression in breast carcinoma specimens and the number of stromal T $CD8^+$ lymphocytes, intratumoral DCs, and stromal NK cells (Figure 1). Consistent with these results and the antitumoral properties of these subpopulations, elevated CX3CL1 expression may be associated with significantly better disease-free survival.

Conversely, T_{reg} infiltration is associated with a poor prognosis. It is now clear that T_{regs} induce an immunomodulatory state by producing IL-10 and TGF-β, which inhibit APC maturation and the expression of costimulatory molecules as well as decreasing the cytotoxic potential of NK cells and cytotoxic T lymphocytes. CCL22 is a chemokine that signals through CCR4 and is a chemoattractant for monocytes, dendritic cells, NK cells, and chronically activated T lymphocytes. Recently, CCL22 was linked to T_{reg} infiltration (Figure 1) in gastric, esophageal, and ovarian carcinomas [30–32]. Consistent with its significance in T_{reg} infiltration, breast tumors that lack CCL22 are not infiltrated by the T_{reg} subpopulation [33]. Moreover, in human breast carcinoma cell lines, CCL22 was secreted at low basal levels and upregulated in response to inflammatory signals.

4. The Function of Chemokines in Angiogenesis

Angiogenesis is a hallmark of cancer. The resulting tumor-associated neovasculature that is generated addresses the tumor's growing demands for nutrients and oxygen [34] and enables the tumor to grow and avoid excessive necrosis.

The CXC chemokine family comprises angiogenic and antiangiogenic chemokines (Table 1). Angiogenic chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8, are generally distinguished by an ELR motif. These chemokines mediate their angiogenicity through CXCR2 and interact alone or with other angiogenic factors (e.g., VEGF) to effect angiogenesis (Figure 2) [26]. CXCL8 is considered one of the most potent inducers of angiogenic processes [12] such as rapid stress fiber assembly, chemotaxis, enhanced proliferation of and tube formation by endothelial cells [26]. Noteworthy, Haim and coworkers [35] reported that estrogen upregulates the transcription and secretion of CXCL8 in

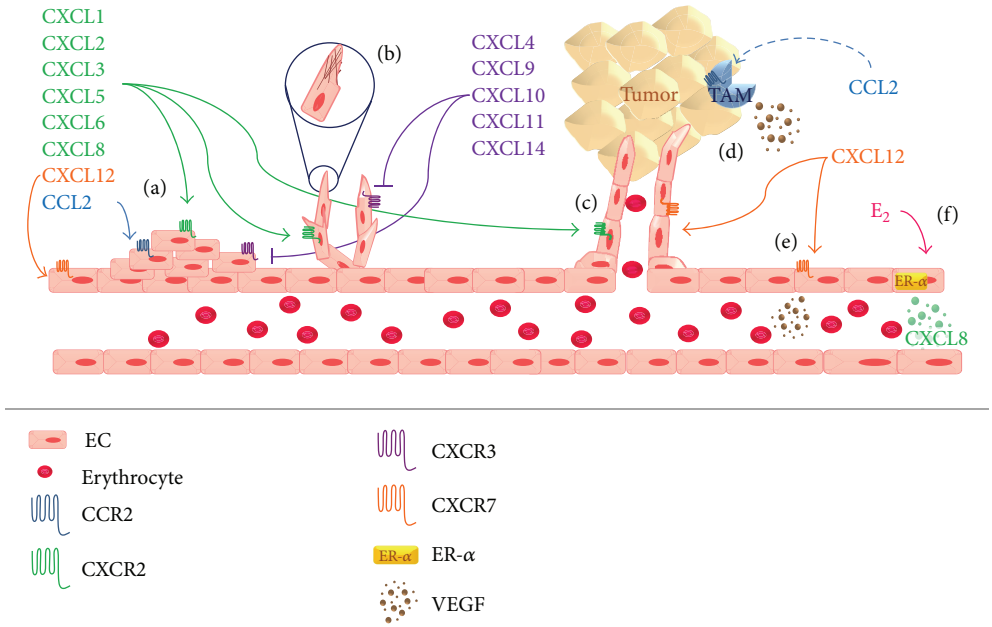

Figure 2: Chemokines involvement in angiogenesis. Angiogenic ELR$^+$ chemokines act through CXCR2 receptor to promote (a) proliferation of endothelial cells (EC), (b) stress fibre assembly, and (c) tube formation. On its behalf, antiangiogenic non-ELR$^+$ chemokines, via CXCR3, inhibit these processes. CCL2 also promotes angiogenesis via CCR2, (a) stimulating EC proliferation and (d) in an indirect manner by increasing TAM infiltration, which secrete angiogenic factors like VEGF. CXCL12 acts through CXCR7 to promote (a) EC proliferation and (e) VEGF production by these cells. It has been reported that (f) estrogen (E_2) stimulates EC secretion of CXCL8.

Table 1: Angiogenic and antiangiogenic members of CXC chemokine family.

Angiogenic ELR$^+$ chemokines	Antiangiogenic non-ELR$^+$ chemokines
CXCL1	CXCL4
CXCL2	CXCL9
CXCL3	CXCL10
CXCL5	CXCL11
CXCL6	CXCL14
CXCL7	
CXCL8	

breast tumor cells additively through estrogen receptor α (ERα), adding a novel role of estrogen in promoting tumor growth (Figure 2).

Although CXCL12 is a non-ELR-CXC chemokine, it has been implicated as an angiogenic chemokine based on evidence of its involvement in blood vessel formation, inducing endothelial cell migration and proliferation, stimulating tube formation, and enhancing VEGF release (Figure 2) [12].

Angiostatic CXC chemokine family members include CXCL4, CXCL9, CXCL10, CXCL11, and CXCL14. CXCL4, CXCL9, and CXCL10 signal through CXCR3, which, on ligand engagement, blocks microvascular endothelial cell migration and proliferation (Figure 2) in response to various angiogenic factors [26].

CCL2 and CCL5 have been also suggested to shift the balance in the tumor microenvironment towards increased vascularity. CCL2 acts directly on endothelial cells to promote angiogenesis and correlates closely with positive endothelial growth regulators, such as vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), and CXCL8 [20]. In an indirect manner, CCL2 increases the presence of TAMs, which produce other angiogenic factors [11] (Figure 2).

In addition to the development of new blood vessels, tumors also undergo a process denominated Lymphangiogenesis—the growth of lymphatic vessels—which contributes to lymphatic metastasis [36] and is thus a major event in the development and spread of cancer. Peritumoral lymphangiogenesis involves the secretion of VEGF-C and VEGF-D, which act on the lymphatic endothelium and are upregulated in the MCF10 breast cancer cell line [37]. Further, CXCL12 is a chemoattractant for lymphangiogenic endothelial cells (LECs), inducing the migration and tubule formation of LECs *in vitro* and lymphangiogenesis *in vivo* and correlating with lymphatic vessel density in cancer tissues [36].

5. The Function of Chemokines in Metastasis

Metastasis is the dissemination of cancer cells to distant organs and tissues, such as the liver, lung, brain, and bone. This process is the most devastating attribute of cancer and significantly influences its morbidity and mortality [38]. Cancer metastasis is not a fortuitous or randomly driven process but is governed by many factors that, for example, allow cancer cells to move, detach from the ECM (which is achieved by the expression of matrix metalloproteases

and heparanase), intravasate, migrate to distant organs and be able to fluorish in a different niche from the one in which they developed. How other cellular populations in the tumor microenvironment contribute to ECM remodeling is beyond the scope of this section, but notably cancer-associated fibroblasts (CAFs) have a significant function in this process.

The CXCL12-CXCR4 axis is one of the most extensively studied pairs in metastasis, primarily with regard to its involvement in organ-directed metastasis. Its function in metastasis begins with cancer cell mobility—the binding of CXCL12 to CXCR4 activates various intracellular signal transduction pathways and effector molecules that regulate chemotaxis, migration, and adhesion. Low-CXCR4-expressing MCF-7 cells fail to metastasize when injected into mice, whereas CXCR4-high MDA-231 cells are efficient in forming distant organ metastases [39]. Similarly, CCL21, through its receptor CCR7, triggers actin polymerization, pseudopodia formation, and the directional migration and invasion of breast cancer cells, particularly to lymph nodes, where CCL21 is highly expressed [40].

CXCR4 expression is higher in malignant breast tumor compared with its normal counterpart [40]. It controls chemotaxis toward its ligand, CXCL12, which is highly expressed in the lung, bone, liver, and lymph nodes, organs to which breast cancer cells preferentially metastasizes [41, 42]. With regard to brain metastasis, it remains unknown how cancer cells breach the brain-blood barrier (BBB) and invade this tissue, but it is possible that CXCL12-expressing CNS cells are chemoattractants for metastatic breast cancer cells [43].

Triple-negative breast cancer (TNBC) is a very aggressive subtype with few therapeutic alternatives and a poor prognosis. CXCR4 expression was recently reported to be more frequent in TNBC versus other subtypes, and CXCR4-positive patients had a significantly higher rate of metastasis, larger primary tumors, and shorter overall- and disease-free survival [44].

Chemokines that are expressed by osteoblasts and bone marrow endothelial cells have been implicated in driving bone metastasis. During their differentiation into osteoblasts, mesenchymal stem cells secrete CCL2, which is believed to mediate the migration of cancer cells, a process that is partially inhibited by anti-CCL2 [45]. CX3CL1 is expressed in a membrane-bound form and is exposed to the luminal side of human bone marrow endothelial cells, whereas its receptor, CX3CR1, is expressed in normal and malignant mammary glands [46]. Breast cancer cells that express high levels of CX3CR1 have a greater propensity toward bone metastasis; consistent with these data, studies in CX3CL1-null transgenic mice suggest that the absence of this chemokine impairs the dissemination of cancer cells to bone [46].

Notably, Hernandez et al. [47] reported that the binding of CXCL12 to CXCR4 and CXCR7 elicits disparate cellular responses. CXCR4 controls chemotactic and invasive behavior (*in vivo* motility and intravasation) in response to CXCL12, whereas CXCR7 enhances primary tumor growth and angiogenesis but decreases *in vivo* invasion, intravasation, and metastasis formation.

As discussed, CCL2 mediates TAM infiltration and generates an amplification loop, upregulating CCL2 in TAMs, which is associated with the expression of membrane type 1-matrix metalloprotease (MT1-MMP) [11]. Similarly, other chemokines and receptors, such as CCL5, CCL20 (via CCR6), CXCL12, and CXCR7, induce or increase the expression of MMPs [12, 14, 47]. CXCL7 has been reported to be linked to greater heparanase activity in MCF-7 breast cancer cells [37].

Recently, Chen and colleagues [48] linked TAM-produced CCL18 with cancer cell invasiveness and identified PITPNM3 (a membrane-associated phosphatidylinositol transfer protein) as its receptor. In this report, CCL18 colocalized with CD68 (a TAM marker) in most invasive breast carcinoma samples. In *in vitro* experiments, the group showed that the invasiveness of MDA-MB-231 cells and primary breast cancer cells was enhanced equally by the addition of recombinant CCL18 and coculture with TAMs, whereas the addition of anti-CCL18 and CCL18-siRNAs reduced the number of invasive cancer cells. Based on these findings, it is concluded that TAMs are a source of CCL18 and there may be a close relationship between CCL18 and invasiveness.

6. Therapeutic Targeting of Chemokines in Breast Cancer

Chemoresistance is a significant obstacle in cancer treatment, because cancer cell subpopulations that survive chemotherapy can proliferate and reemerge as a more aggressive variant, limiting subsequent therapeutic options. CCL25, via CCR9, confers a survival advantage to breast cancer cells by inhibiting cisplatin-induced apoptosis in a PI3K-dependent manner, in addition to activating cell survival signals through Akt [49]. Acharyya et al. reported a notable feedback mechanism between chemotherapy treatment and chemokine-induced chemoresistance [50], in which CXCL1 and CXCL2 attract $CD11b^{+}GR1^{+}$ myeloid cells, which produce other chemokines, including S100A8/9, that enhance cancer cell survival. Although chemotherapeutic agents kill cancer cells, they induce TNF-α production by endothelial and stromal cells, which upregulates CXCL1 and CXCL2 in cancer cells, amplifying the CXCL1/2-S100A8/9 loop and affecting chemoresistance.

Chemokines and chemokine receptors are appealing targets for cancer treatment, based on the wide range of processes that they influence. For instance, chemokine receptors mediate critical steps in the development and spread of cancer, for which antagonists have been designed to inhibit signal transduction and impede the undesired effects of chemokines. In CXCL1- and CXCL2-induced chemoresistance, CXCR2 blockers have been shown to break the chemokine-chemoresistance cycle described above, augmenting the efficacy of chemotherapy [50].

As described, CCL5 mediates cancer cell invasiveness and signals through CCR5. CCR5 antagonists slow the invasion of basal breast carcinoma cells *in vitro* and decrease pulmonary metastasis in a preclinical mouse model of breast cancer,

suggesting that CCR5 antagonists can be used as adjuvant therapy in patients with this breast cancer subtype [51].

Concerning the chemoresistant CSC population, it is known that chemotherapy induces the expression of CXCL8 in injured cells, which increases the activity and self-renewal of the former. Thus, the blockade of CXCL8 receptors CXCR1 and CXCR2 arises as a promising side-therapy attempting to avoid tumor recurrence [16, 52]. Furthermore, not only the number of CSCs but also a reduction in general tumor cell viability is achieved by the use of CXCR1 inhibitors [52].

Because the CXCL12-CXCR4 axis has significant function in breast cancer metastasis, it has also been targeted using CXCR4 antagonists. Williams and colleagues [27] reported that a CXCL12 analog with antagonist activity (CXCL12(P2G)) significantly inhibited metastasis in a syngeneic mouse model of breast carcinoma. Conversely, T140 analogs are peptidic CXCR4 antagonists, originally developed as anti-HIV agents that inhibit CXCL12-induced migration of MDA-MB-231 breast cancer cells *in vitro* and mitigate pulmonary metastasis *in vivo* [53].

Chemokines that promote and enhance the activity and interaction of immune cells have been exploited as a prophylactic approach. CCL19 and CCL21 regulate the encounter between DCs and T lymphocytes in lymph nodes, for which they can be considered as important natural adjuvants for immune response [54, 55]. These chemokines have been used in DNA vaccines, amplifying their immunogenicity, inducing a Th1-polarized immune response, and substantially improving their protective effects in BALB/c mice [55]. Oncolytic viruses are also promising cancer treatments; this virotherapy, in combination with a CXCR4 antagonist, has increased efficacy over oncolysis alone in a triple-negative breast carcinoma syngeneic mouse model; systemic delivery of the armed virus after resection of the primary tumor inhibits the development of metastasis and increases overall tumor-free survival [56].

7. Concluding Remarks

Rapidly accumulating data in breast cancer immunology from recent years suggest that many established and widely accepted paradigms should be revised. In breast cancer, whereas chemokines are primarily associated with the generation of a protumoral microenvironment and organ-directed metastasis, they also appear to mediate disease progression, favoring the growth and proliferation of tumor cells.

Recent studies suggest that inhibiting local chemokines signaling in the tumor by blocking particular receptors or using analogs with antagonist activity could be a new rationale promising strategy for controlling tumor development and progression. Thus, the development of drugs that specifically target the chemokine axis will be invaluable in the treatment of breast cancer, in which inflammation has a major role.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] M. Locati, K. Otero, T. Schioppa et al., "The chemokine system: Tuning and shaping by regulation of receptor expression and coupling in polarized responses," *Allergy*, vol. 57, no. 11, pp. 972–982, 2002.

[2] A. Rot and U. H. von Andrian, "Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells," *Annual Review of Immunology*, vol. 22, pp. 891–928, 2004.

[3] S. J. Allen, S. E. Crown, and T. M. Handel, "Chemokine: receptor structure, interactions, and antagonism," *Annual Review of Immunology*, vol. 25, pp. 787–820, 2007.

[4] J. Ferlay, I. Soerjomataram, M. Ervik et al., *LOBOCAN 2012 v1.0: Cancer Incidence and Mortality Worldwide: IARC CancerCase No. 11*, 2013.

[5] S. B. Edge and C. C. Compton, "The American joint committee on cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM," *Annals of Surgical Oncology*, vol. 17, no. 6, pp. 1471–1474, 2010.

[6] S. M. Burnet, "Cancer-a biological approach," *British Medical Journal*, vol. 1, no. 5023, pp. 841–847, 1957.

[7] M. J. Smyth, G. P. Dunn, and R. D. Schreiber, "Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity," *Advances in Immunology*, vol. 90, pp. 1–50, 2006.

[8] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The immunobiology of cancer immunosurveillance and immunoediting," *Immunity*, vol. 21, no. 2, pp. 137–148, 2004.

[9] S. Ali and G. Lazennec, "Chemokines: novel targets for breast cancer metastasis," *Cancer and Metastasis Reviews*, vol. 26, no. 3-4, pp. 401–420, 2007.

[10] S. L. Deshmane, S. Kremlev, S. Amini, and B. E. Sawaya, "Monocyte chemoattractant protein-1 (MCP-1): an overview," *Journal of Interferon and Cytokine Research*, vol. 29, no. 6, pp. 313–326, 2009.

[11] G. Soria and A. Ben-Baruch, "The inflammatory chemokines CCL2 and CCL5 in breast cancer," *Cancer Letters*, vol. 267, no. 2, pp. 271–285, 2008.

[12] A. Ben-Baruch, "The multifaceted roles of chemokines in malignancy," *Cancer and Metastasis Reviews*, vol. 25, no. 3, pp. 357–371, 2006.

[13] A. Tsuyada, A. Chow, J. Wu et al., "CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells," *Cancer Research*, vol. 72, no. 11, pp. 2768–2779, 2012.

[14] S. Marsigliante, C. Vetrugno, and A. Muscella, "CCL20 induces migration and proliferation on breast epithelial cells," *Journal of Cellular Physiology*, vol. 228, no. 9, pp. 1873–1883, 2013.

[15] N. Shao, L.-H. Chen, R.-Y. Ye, Y. Lin, and S.-M. Wang, "The depletion of Interleukin-8 causes cell cycle arrest and increases the efficacy of docetaxel in breast cancer cells," *Biochemical and Biophysical Research Communications*, vol. 431, no. 3, pp. 535–541, 2013.

[16] J. K. Singh, G. Farnie, N. J. Bundred et al., "Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms," *Clinical Cancer Research*, vol. 19, no. 3, pp. 643–656, 2013.

[17] L. V. Rhodes, S. P. Short, N. F. Neel et al., "Cytokine receptor CXCR4 mediates estrogen-independent tumorigenesis, metastasis, and resistance to endocrine therapy in human breast cancer," *Cancer Research*, vol. 71, pp. 603–613, 2011.

[18] X.-L. Gu, Z.-L. Ou, F.-J. Lin et al., "Expression of CXCL14 and its anticancer role in breast cancer," *Breast Cancer Research and Treatment*, vol. 135, no. 3, pp. 725–735, 2012.

[19] H. Fujimoto, T. Sangai, G. Ishii et al., "Stromal MCP-1 in mammary tumors induces tumor-associated macrophage infiltration and contributes to tumor progression," *International Journal of Cancer*, vol. 125, no. 6, pp. 1276–1284, 2009.

[20] T. Ueno, M. Toi, H. Saji et al., "Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer," *Clinical Cancer Research*, vol. 6, no. 8, pp. 3282–3289, 2000.

[21] S. C. Robinson, K. A. Scott, J. L. Wilson, R. G. Thompson, A. E. I. Proudfoot, and F. R. Balkwill, "A chemokine receptor antagonist inhibits experimental breast tumor growth," *Cancer Research*, vol. 63, no. 23, pp. 8360–8365, 2003.

[22] E. P. Adler, C. A. Lemken, N. S. Katchen, and R. A. Kurt, "A dual role for tumor-derived chemokine RANTES (CCL5)," *Immunology Letters*, vol. 90, no. 2-3, pp. 187–194, 2003.

[23] L. F. Neville, G. Mathiak, and O. Bagasra, "The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily," *Cytokine and Growth Factor Reviews*, vol. 8, no. 3, pp. 207–219, 1997.

[24] J. M. Farber, "Mig and IP-10: CXC chemokines that target lymphocytes," *Journal of Leukocyte Biology*, vol. 61, no. 3, pp. 246–257, 1997.

[25] R. M. Strieter, J. A. Belperio, R. J. Phillips, and M. P. Keane, "CXC chemokines in angiogenesis of cancer," *Seminars in Cancer Biology*, vol. 14, no. 3, pp. 195–200, 2004.

[26] R. M. Strieter, M. D. Burdick, J. Mestas, B. Gomperts, M. P. Keane, and J. A. Belperio, "Cancer CXC chemokine networks and tumour angiogenesis," *European Journal of Cancer*, vol. 42, no. 6, pp. 768–778, 2006.

[27] S. A. Williams, Y. Harata-Lee, I. Comerford, R. L. Anderson, M. J. Smyth, and S. R. McColl, "Multiple functions of CXCL12 in a syngeneic model of breast cancer," *Molecular Cancer*, vol. 9, article 250, 2010.

[28] G. E. White and D. R. Greaves, "Fractalkine: a survivor's guide—chemokines as antiapoptotic mediators," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 3, pp. 589–594, 2012.

[29] M. H. Park, J. S. Lee, and J. H. Yoon, "High expression of CX3CL1 by tumor cells correlates with a good prognosis and increased tumor-infiltrating CD8+ T cells, natural killer cells, and dendritic cells in breast carcinoma," *Journal of Surgical Oncology*, vol. 106, no. 4, pp. 386–392, 2012.

[30] Y. Mizukami, K. Kono, Y. Kawaguchi et al., "CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3$^+$ regulatory T cells in gastric cancer," *International Journal of Cancer*, vol. 122, no. 10, pp. 2286–2293, 2008.

[31] T. Maruyama, K. Kono, S. Izawa et al., "CCL17 and CCL22 chemokines within tumor microenvironment are related to infiltration of regulatory T cells in esophageal squamous cell carcinoma," *Diseases of the Esophagus*, vol. 23, no. 5, pp. 422–429, 2010.

[32] T. J. Curiel, G. Coukos, L. Zou et al., "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival," *Nature Medicine*, vol. 10, no. 9, pp. 942–949, 2004.

[33] J. Faget, C. Biota, T. Bachelot et al., "Early detection of tumor cells by innate immune cells leads to T reg recruitment through CCL22 production by tumor cells," *Cancer Research*, vol. 71, no. 19, pp. 6143–6152, 2011.

[34] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, 2011.

[35] K. Haim, P. Weitzenfeld, T. Meshel, and A. Ben-Baruch, "Epidermal growth factor and estrogen act by independent pathways to additively promote the release of the angiogenic chemokine CXCL8 by breast tumor cells," *Neoplasia*, vol. 13, no. 3, pp. 230–243, 2011.

[36] W. Zhuo, L. Jia, N. Song et al., "The CXCL12 -CXCR4 chemokine pathway: a novel axis regulates lymphangiogenesis," *Clinical Cancer Research*, vol. 18, no. 19, pp. 5387–5398, 2012.

[37] M. Yu, R. Berk, and M. A. Kosir, "CXCL7-mediated stimulation of lymphangiogenic factors VEGF-C, VEGF-D in human breast cancer cells," *Journal of Oncology*, vol. 2010, Article ID 939407, 10 pages, 2010.

[38] Z. Liang, W. Zhan, A. Zhu et al., "Development of a unique small molecule modulator of cxcr4," *PLoS ONE*, vol. 7, no. 4, Article ID e34038, 2012.

[39] M. Z. Dewan, S. Ahmed, Y. Iwasaki, K. Ohba, M. Toi, and N. Yamamoto, "Stromal cell-derived factor-1 and CXCR4 receptor interaction in tumor growth and metastasis of breast cancer," *Biomedicine and Pharmacotherapy*, vol. 60, no. 6, pp. 273–276, 2006.

[40] A. Müller, B. Homey, H. Soto et al., "Involvement of chemokine receptors in breast cancer metastasis," *Nature*, vol. 410, no. 6824, pp. 50–56, 2001.

[41] D. Mukherjee and J. Zhao, "The Role of chemokine receptor CXCR4 in breast cancer metastasis," *American Journal of Cancer Research*, vol. 3, pp. 46–57, 2013.

[42] M. Kucia, R. Reca, K. Miekus et al., "Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis," *Stem Cells*, vol. 23, no. 7, pp. 879–894, 2005.

[43] C. V. Hinton, S. Avraham, and H. K. Avraham, "Role of the CXCR4/CXCL12 signaling axis in breast cancer metastasis to the brain," *Clinical and Experimental Metastasis*, vol. 27, no. 2, pp. 97–105, 2010.

[44] H.-W. Chen, C.-W. Du, X.-L. Wei, U.-S. Khoo, and G.-J. Zhang, "Cytoplasmic CXCR4 high-expression exhibits distinct poor clinicopathological characteristics and predicts poor prognosis in triple-negative breast cancer," *Current Molecular Medicine*, vol. 13, no. 3, pp. 410–416, 2013.

[45] A. P. Molloy, F. T. Martin, R. M. Dwyer et al., "Mesenchymal stem cell secretion of chemokines during differentiation into osteoblasts, and their potential role in mediating interactions with breast cancer cells," *International Journal of Cancer*, vol. 124, no. 2, pp. 326–332, 2009.

[46] W. L. Jamieson-Gladney, Y. Zhang, A. M. Fong, O. Meucci, and A. Fatatis, "The chemokine receptor CX3CR1 is directly involved in the arrest of breast cancer cells to the skeleton," *Breast Cancer Research*, vol. 13, no. 5, article R91, 2011.

[47] L. Hernandez, M. A. O. Magalhaes, S. J. Coniglio, J. S. Condeelis, and J. E. Segall, "Opposing roles of CXCR4 and CXCR7 in breast cancer metastasis," *Breast Cancer Research*, vol. 13, no. 6, article R128, 2011.

[48] J. Chen, Y. Yao, C. Gong et al., "CCL18 from Tumor-Associated Macrophages Promotes Breast Cancer Metastasis via PITPNM3," *Cancer Cell*, vol. 19, no. 4, pp. 541–555, 2011.

[49] C. Johnson-Holiday, R. Singh, E. L. Johnson, W. E. Grizzle, J. W. Lillard Jr., and S. Singh, "CCR9-CCL25 interactions promote

cisplatin resistance in breast cancer cell through Akt activation in a PI3K-dependent and FAK-independent fashion," *World Journal of Surgical Oncology*, vol. 9, article 43, 2011.

[50] S. Acharyya, T. Oskarsson, S. Vanharanta et al., "A CXCL1 paracrine network links cancer chemoresistance and metastasis," *Cell*, vol. 150, no. 1, pp. 165–178, 2012.

[51] M. Velasco-Velázquez, X. Jiao, M. De La Fuente et al., "CCR5 antagonist blocks metastasis of basal breast cancer cells," *Cancer Research*, vol. 72, no. 15, pp. 3839–3850, 2012.

[52] C. Ginestier, S. Liu, M. E. Diebel et al., "CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts," *Journal of Clinical Investigation*, vol. 120, no. 2, pp. 485–497, 2010.

[53] H. Tamamura, A. Hori, N. Kanzaki et al., "T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer," *FEBS Letters*, vol. 550, no. 1–3, pp. 79–83, 2003.

[54] T. Nguyen-Hoai, G. Baldenhofer, M. S. Ahmed et al., "CCL19 (ELC) improves TH1-polarized immune responses and protective immunity in a murine Her2/neu DNA vaccination model," *Journal of Gene Medicine*, vol. 14, no. 2, pp. 128–137, 2012.

[55] T. Nguyen-Hoai, G. Baldenhofer, M. S. Sayed Ahmed et al., "CCL21 (SLC) improves tumor protection by a DNA vaccine in a Her2/neu mouse tumor model," *Cancer Gene Therapy*, vol. 19, no. 1, pp. 69–76, 2012.

[56] M. Gil, M. Seshadri, M. P. Komorowski, S. I. Abrams, and D. Kozbor, "Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 14, pp. E1291–E1300, 2013.

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The Role of Cytokines in Breast Cancer Development and Progression

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Cytokines are highly inducible, secretory proteins that mediate intercellular communication in the immune system. They are grouped into several protein families that are referred to as tumor necrosis factors, interleukins, interferons, and colony-stimulating factors. In recent years, it has become clear that some of these proteins as well as their receptors are produced in the organisms under physiological and pathological conditions. The exact initiation process of breast cancer is unknown, although several hypotheses have emerged. Inflammation has been proposed as an important player in tumor initiation, promotion, angiogenesis, and metastasis, all phenomena in which cytokines are prominent players. The data here suggest that cytokines play an important role in the regulation of both induction and protection in breast cancer. This knowledge could be fundamental for the proposal of new therapeutic approaches to particularly breast cancer and other cancer-related disorders.

Introduction

The process by which breast cancer is initiated is unknown, for which several hypotheses have emerged. Inflammation has been proposed to mediate the initiation and promotion of tumors, angiogenesis, and metastasis (Grivennikov and others 2010). Inflammatory cells are attracted by oncogenic changes, hypoxia, cytokines, and chemokines, among other factors. Inflammation in a tumor microenvironment comprises infiltrating immune cells and activated fibroblasts that secrete cytokines, chemokines, and growth factors to which the tumor responds (Coussens and Werb 2002; Grivennikov and others 2010).

Obesity can result in an inflammatory environment that can contribute to tumorigenesis. Menopause and increased age are also associated with systemic inflammation (Bruunsgaard and others 2001; Pfeilschifter and others 2002). In turn, cancer therapy can effect an inflammatory tumor microenvironment by provoking extensive tumor cell death (Baumgarten and Frasor 2012).

Several cytokines regulate the inflammatory tumor microenvironment. Interleukin (IL)-1, IL-6, IL-11, and transforming growth factor-β (TGF-β) stimulate cancer cell proliferation and invasion (Nicolini and others 2006), and cytokine receptor activation and intracellular signaling by NF-κB accelerate tumor progression (Karin and Greten 2005; Hsing and others 2012).

Transforming growth factor-β

TGF-β is the most extensively studied cytokine in breast cancer. TGF-β belongs to the TGF-β superfamily and is a major regulator of many processes, including proliferation, differentiation, migration, immunity, and apoptosis. TGF-β has dual functions in tumor progression. As a tumor suppressor, it has antiproliferative effects in the early stages of tumorigenesis, but tumor cells in later stages evade this effect and progress in response to TGF-β (Fig. 1) (Joshi and Cao 2010; Band and Laiho 2011; Inman 2011; Meulmeester and Ten Dijke 2011; Zu and others 2012). TGF-β, TβRII (the receptor required for TGF-β signaling), and phospho-Smad2 expression are associated with earlier age of onset and aggressive tumor characteristics (Figueroa and others 2010).

In the early stages of cancer, TGF-β causes cell-cycle arrest, particularly in epithelial, endothelial, and hematopoietic cells (Massagué 2008; Heldin and others 2009; Tian and Schiemann 2010; Allington and Schiemann 2011), inhibiting cyclin-dependent kinases by downregulating c-Myc and ID1 and upregulating CDK inhibitors, including p15 and p21 (Donovan and Slingerland 2000; Feng and others 2002; Perk and others 2005; Glasgow and Mishra 2008; Massagué 2008; Juárez and Guise 2010). TGF-β also restricts estrogen receptor (ER)α-mediated proliferation (Ewan and others 2005; Band and Laiho 2011). Many triple-negative human breast cancer cell lines, including MDA-MB-231,

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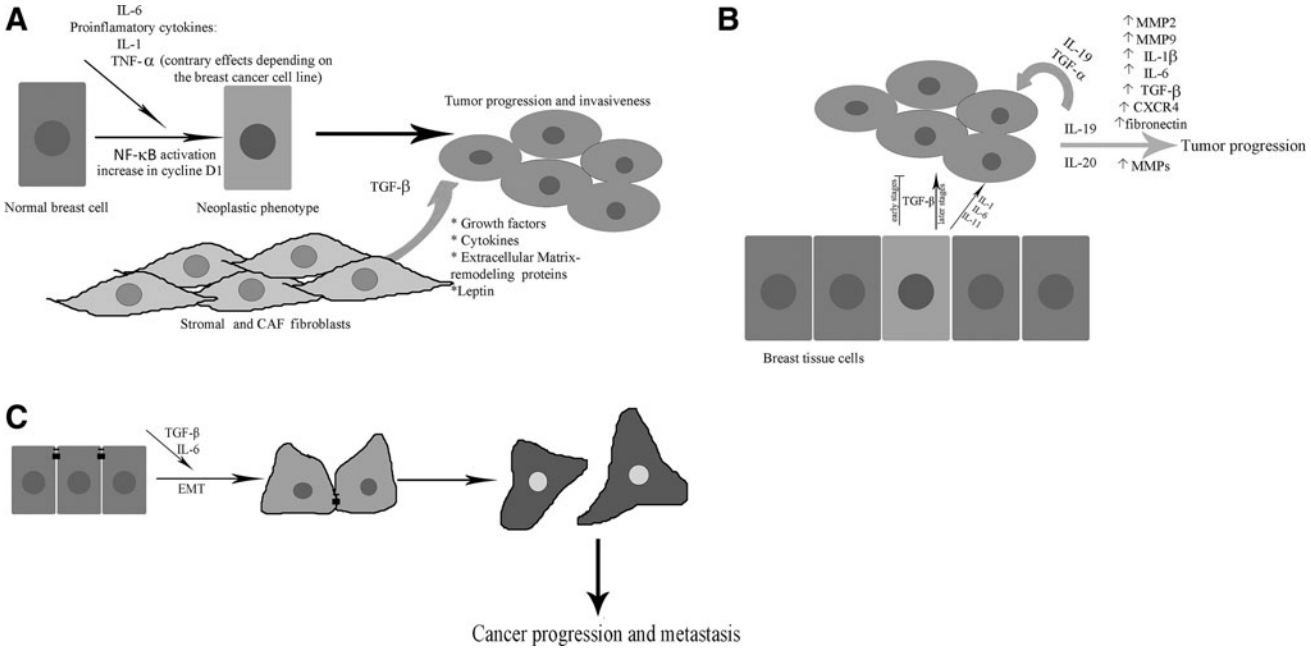

FIG. 1. Role of cytokines in the different stages of breast cancer. This figure resumes the role that different cytokines have on establishment, progression, and metastasis of breast cancer. **(A)** Normal cells can adopt a neoplastic phenotype by the action of IL-6, IL-1, TNF-α, and proinflammatory cytokines, which cause NF-κB activation and an increase in cycline D1 in the normal breast cell, resulting in a neoplastic phenotype. **(B)** The proliferation of these cells, at early stages of cancer, is suppressed by TGF-β. However, at later stages, TGF-β promotes proliferation of these cells, tumor progression, and invasiveness. **(C)** Stromal fibroblasts and CAFs are an important source of TGF-β. TGF-β and IL-6 can lead to the initiation of the EMT process, which finally ends in cells that can survive not bonded to other cells and, thus, can migrate to other parts of the body and result in the spreading of the cancer.

BT20, Hs578T, HCC1937, and HCC3153, are resistant to the growth-inhibitory activity of TGF-β (Lin and others 2012). In many cases, the antiproliferative effects of TGF-β are attributed to altered TGF-β signaling due to somatic mutations in components of TGF-β signaling or selective inhibition of cytostatic responses to TGF-β. Mutations in TβR-I are infrequently detected in breast cancers (Imamura and others 2012).

The tumor-promoting effects of TGF-β are complex and entail the expression and translocation of the nuclear factor of activated T cells into the nucleus, stimulating c-Myc expression (Singh and others 2010). TGF-β activates Smad3/4, which, in turn, specifically binds to the *HDM2* promoter and upregulates *HDM2,* destabilizing p53 in human breast cancer (Zu and others 2012).

TGF-β is a mediator of the epithelial-to-mesenchymal transition (EMT), which is categorized into 4 subtypes: type 1, or embryonic and developmental EMT; type 2, or tissue-regeneration and fibrotic EMT; and type 3, or cancer progression and metastatic EMT (Kalluri and Weinberg 2009). Type 3 EMT has been linked to the progression and dissemination of breast cancer. TGF-β generates polarized epithelial cells that alter their epithelial phenotype by downregulating genes which form adherent and tight junctions, remodel the cytoskeleton, and upregulate genes that are associated with cell motility and a mesenchymal phenotype (Heldin and others 2009; Wendt and others 2009; Xu and others 2009; Allington and Schiemann 2011; Zu and others 2012), promoting tumor growth and metastasis (Vincent and others 2009; Voulgari and Pintzas 2009; Wendt and others 2009; Xie and others 2012).

The mechanisms through which TGF-β promotes the EMT are complex. For example, in Wnt signaling, the transcriptional repressor SNAIL1 affects the EMT through its interaction with Smad3 and Smad4, which downregulates CAR, occludin, claudin-3, and E-cadherin in breast epithelial cells (Vincent and others 2009). TGF-β also upregulates TF3, a transcription factor that regulates morphology, EMT marker expression, and cancer-initiating features in breast cells (Yin and others 2010). TGF-β communicates with Wnt, Her2, and FAK, which influence the EMT and breast cancer stem cells (BCSCs), and it promotes the formation of cancer stem cells (Taube and others 2010; Jain and Alahari 2011; Zu and others 2012).

Interleukin-6

Breast cancer cell lines produce IL-6, of which ER-positive cells secrete lower levels than ER-negative cells. IL-6 induces proliferation and a more aggressive phenotype in ER-positive cells (Sasser and others 2007). Fibroblasts from breast tissue also secrete IL-6, which stimulates the growth and invasiveness of MCF-7 cells (Studebaker and others 2008; Baumgarten and Frasor 2012). Further, IL-6 regulates the inducible formation and maintenance of BCSCs (Iliopoulos and others 2011). Through the IL-6 receptor/GP130 complex and STAT3 activation, IL-6 governs the self-renewal of BCSCs (Iliopoulos and others 2009, 2010; Korkaya and others 2011). Overexpression of IL-6 in MCF-7 cells induces the EMT and increases their invasiveness (Sullivan and others 2009).

IL-6 bridges Stat3 and NF-κB-dependent inflammatory cytokines (eg, IL-1, TNF-α). The initial activation of NF-κB by inflammatory signals activates a self-reinforcing regulatory circuit that comprises IL-6 and Stat3 and converts a stable normal cellular phenotype into a stable neoplastic phenotype without any change in DNA sequence (Iliopoulos and others 2009), linking tumorigenesis to NF-κB activation and inflammation (Ernst and Putoczki 2012).

Tumor necrosis factor-α

Tumor necrosis factor (TNF)-α, an inflammatory cytokine that is highly expressed in breast carcinomas (Leek and others 1998), stimulates the proliferation of T47D cells through an NF-κB-dependent increase in cyclin D1 (Baumgarten and Frasor 2012). Inhibition of NF-κB and TNF-α is protective against chemically induced breast tumorigenesis (Connelly and others 2011). Further, *in vitro* activation of the TNF-α/NF-κB axis induces invasive and malignant behavior in breast cancer cells (Balkwill 2009). Chronic expression of TNF-α in breast tumors supports tumor growth (Kamel and others 2012), but the effects of TNF-α *in vitro* differ between breast cancer cell lines. In contrast to its effects in T47D cells, TNF-α induces apoptosis (Donato and Klostergaard 2004); inhibits proliferation; and promotes migration, invasion, and resistance to chemotherapeutic drugs in MCF-7 cells (Goldberg and Schwertfeger 2010), regulating genes and enzymes that mediate estrogen metabolism, leading to higher levels of DNA adducts (Kamel and others 2012). Thus, TNF-α is a necrotic and tumor-promoting factor—in the tumor microenvironment, TNF-α enhances tumor growth and migration, whereas local administration of high doses of TNF-α has robust antiangiogenic and antitumoral effects (Hamed and others 2012).

Interleukin-17

IL-17, a cytokine that is secreted by CD4 and CD8 cells (6–8), is required for the development and tumor-promoting activity of myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice (He and others 2010). TGF-β, IL-6, and IL-23 have been implicated in the initiation of Th17 cell differentiation in mice (Veldhoen and others 2006; Zhou and others 2007; Novitskiy and others 2011).

IL-19, IL-20, TGF-α, and IL-23

IL-19, IL-20, TGF-α, and IL-23 are also involved in breast tumorigenesis and tumor progression. IL-19 provides a microenvironment that is conducive to tumor progression through an autocrine effect, stimulating the proliferation and migration of cancer cells through matrix metalloproteinase (MMP)-2, MMP-9, IL-1β, IL-6, TGF-β, CXCR4, and fibronectin (Hsing and others 2012). Studies *in vitro* have demonstrated that IL-19 induces the proliferation of the MCF-7 and Hs578T human breast carcinoma cell lines and of the 67NR and 4T1 murine breast cancer lines (Hsing and others 2012). IL-20 enhances the proliferation and migration of cancer cells and creates a microenvironment that fosters tumor progression by upregulating MMPs and cathepsins (Hsu and others 2012). In turn, IL-23 affects inflammation and angiogenesis in the tumor microenvironment while tempering $CD8^+$ T-cell infiltration (Langowski and others 2006). TGF-α promotes tumor growth and progression through an autocrine/paracrine loop that involves EGFR (Ziober and others 1993; Humphreys and Hennighausen 2000; Booth and Smith 2007).

Adipokines and Breast Cancer

Obesity is a significant risk factor for breast cancer development. Obesity is associated with elevated levels of proinflammatory cytokines in adipose tissue and in circulation, which establishes a low-grade, chronic inflammatory state. One hallmark of obesity-associated inflammation is the recruitment of macrophages into adipose tissue. Macrophages and adipocytes produce inflammatory factors, such as adipokines and cytokines (Ouchi and others 2011), leading to the activation of NF-κB in adipose tissue and the liver (Cai 2009; Baumgarten and Frasor 2012).

Adipokines (cytokines that are secreted by adipose tissue), such as leptin, adiponectin, IL-6, TNF-α, and IL-1, mediate inflammatory diseases and obesity (Tilg and Moschen 2006). Glucose and fatty acids enhance the ability of adipocytes to produce factors, including IL-8, RANTES, and IGF-1, that influence cancer cell phenotypes. Stromal vascular fraction cells and differentiated adipocytes from obese individuals release more IGF-1 than those from lean individuals, suggesting that obesity favors breast cancer cell growth (D'Esposito and others 2012). Leptin synthesis and plasma levels increase with obesity (Wu and others 2009; Barone and others 2012).

In breast biopsies, IL-1 is 1 of the 5 cytokines (with IL-2, IL-4, IL-10, and G-CSF) that are overexpressed in ductal breast carcinoma but undetected in normal breast tissue (Pantschenko and others 2003; Chavey and others 2007). The production of IL-1, even in small amounts, induces potent secondary responses, in part through its ability to elicit the secretion of other cytokines, chemokines, adhesion molecules, and receptors for cytokines from various cells (Dinarello 1996). IL-1 has been linked to the proliferation, invasion, angiogenesis, and inhibition of apoptosis in cancer cells (Apte and others 2006; Lewis and others 2006). IL-1 and IL-8 induce breast cancer progression by enhancing metastasis and cachexia (Wolf and others 2001; Veldhoen and others 2006). IL-1 family members also modulate the activity of estrogens and their receptors—IL-1 expression is primarily observed in ER-negative breast tumors (Miller and others 2000).

IL-1-induced proliferation is mediated by the estrogen-synthesizing enzymes P450 aromatase and steroid sulfatase, which generate bioactive estrogens. IL-1 enhances aromatase activity in SK-BR3 cells and steroid sulfatase activity in MCF-7 cells by 120% and 130%, respectively (Honma and others 2002). IL-1 receptors are expressed in estrogen-dependent (MCF-7, ZR75-1) and estrogen-independent cell lines (MDA-MB 231) (Pantschenko and others 2003). In contrast, IL-1α, IL-1β, and IL-1ra are preferentially expressed in highly malignant and invasive mammary cell lines (BT 20, BT 549, HS 578T, and MDA-MB 231) and not in the MCF-7, T47-D, ZR75-1, or SKBR-3 lines (Singer and others 2003). TNF and IL-6 upregulate aromatase in the tumor microenvironment, which might stimulate the growth of ER-positive cancers (Cleary and Grossmann 2009). TNF and IL-6 expression correlates with aromatase levels in breast cancer but not in the adjacent normal breast tissue (Irahara and others 2006).

Leptin, a proinflammatory cytokine (Otero and others 2006), is secreted primarily by adipocytes, although breast cancer cell lines produce leptin in a process that is regulated by IL-1 (Faggioni and others 1998; O'brien and others 1999; Iguchi and others 2001). Leptin and its receptor, ObR, are expressed in normal breast epithelial cell lines and breast cancer cell lines (O'brien and others 1999; Hu and others 2002; Laud and others 2002). Leptin is expressed in ductal breast carcinoma but not in healthy breast tissues, and its expression correlates with the stage of invasion (Caldefie-Chézet and others 2005; Jardé and others 2008). Further, ObR is present in human breast carcinoma but not in normal breast tissue (Perrier and others 2009). Leptin upregulates aromatase in MCF-7 cells through a greater binding of AP-1 to promoters (Catalano and others 2003). In breast cancer, increased AP-1 levels correlate with high expression of several cytokines, including IL-1β (Chavey and others 2007).

ObR expression is associated with ER and tumor size (Jardé and others 2008), implicating an interaction between the leptin and estrogen systems to promote breast carcinogenesis. Several immune cells express ObRs, which might render them responsive to leptin (Martín-Romero and others 2000; Caldefie-Chezet and others 2001; Fujita and others 2002; Caldefie-Chezet and others 2003; Zhao and others 2003). In rats, elevated IL-1β concentrations in peripheral blood increase leptin levels and total body fat mass and stimulate the growth of mammary epithelium (Reichlin and others 2000).

Cancer cells organize their microenvironment, recruiting stromal fibroblasts in the desmoplasmic reaction; these fibroblasts and α-smooth muscle actin-positive myofibroblasts (MFs)—collectively termed carcinoma-associated fibroblasts (CAFs)—are reprogrammed to produce growth factors, cytokines, and extracellular matrix (ECM)-remodeling proteins that act in an autocrine and paracrine manner to support tumor proliferation and invasion into surrounding tissues (Orimo and Weinberg 2006; Casey and others 2008; Kojima and others 2010; Barone and others 2012; Zu and others 2012) (Fig. 1).

During tumor progression, TGF-β stimulates the progressive conversion of mammary fibroblasts into CAF MFs, promoting tumor progression (Casey and others 2008; Kojima and others 2010; Shangguan and others 2012; Zu and others 2012). Mesenchymal stem cells (MSCs) are a source of CAFs that are phenotypically similar to MFs (Ostman and Augsten 2009). When injected with cancer cells, MSCs promote the growth and metastasis of cancers (Karnoub and others 2007; Shangguan and others 2012). MSCs are recruited to developing tumors, where they increase breast cancer cell motility, invasion, and metastatic potential by secreting chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES) (Karnoub and others 2007).

In breast tumor development, leptin is a determinant of the tumor-promoting activity of CAFs in normal and K303R-mutated ERα-expressing breast cancer cells, demonstrating that cross-talk exists between breast cancer cells and ''educated'' CAFs which drives tumor progression through leptin signaling (Barone and others 2012). Leptin, secreted from CAFs, binds to its receptor; activates K303R-ERα; and stimulates the proliferation, migration, and invasiveness of K303R-ERα–expressing breast cancer cells. In turn, K303R cells release factors, such as EGF, that induce CAFs to enhance leptin secretion, which might establish a positive feedback loop between cancer and stromal cells to further support breast tumor progression (Barone and others 2012). In addition to CAFs, adipose stromal cells effect invasion and metastasis by MCF-7 cells—a phenotype that is driven by IL-6 (Walter and others 2009; Baumgarten and Frasor 2012).

Adiponectin, another adipokine, might also be involved in breast cancer development. Adiponectin has antiproliferative effects on human breast cancer cells through the initiation of apoptosis and inhibition of the cell cycle (Kang and others 2005; Dieudonne and others 2006; Perrier and others 2009) (Fig. 2).

Observational evidence suggests that the risk for breast cancer decreases with intentional weight loss. Cancer incidence rates have declined in patients who have undergone bariatric surgery, whereas surgery is associated with an 83% lower risk of incident breast cancer. The ratios of percentage weight loss to percentage change in estradiol and SHBG suggest that a 10% loss in body weight affects a reduction in free estradiol levels by at least one-third. Further, a 10% loss in weight is expected to produce decreases in inflammatory markers by one-third. TNF-α and IL-6 levels also decrease with intentional weight loss, albeit to a lesser extent (Byers and Sedjo 2011). Thus, intentional weight loss might be an effective prophylactic method of reducing the risk of breast cancer or a secondary treatment that improves the prognosis of breast cancer patients.

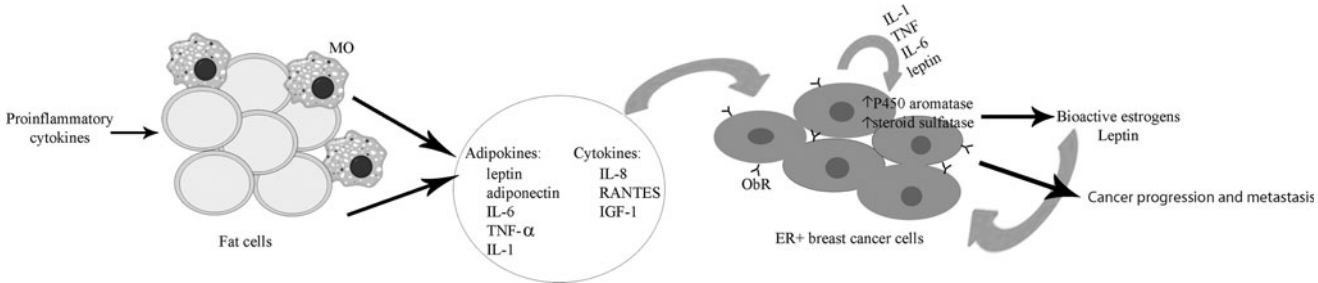

FIG. 2. Role of adipokynes and other cytokines in the progression of breast cancer. Obesity is associated with elevated levels of proinflammatory cytokines in adipose tissue and in circulation, which establishes a low-grade, chronic inflammatory state. Fat cells and macrophages (MO) associated with them produce adipokines and cytokines to which breast cancer cells respond by increasing the expression of P450 aromatase and steroid sulfatase, which, in turn, generate bioactive estrogens; and by producing several cytokines that act in an autocrine fashion. These responses lead to cancer progression and metastasis.

Cytokines and Angiogenesis

Many cytokines participate in angiogenesis, which is essential for tumor growth and progression. TGF-β enhances tumor vascularity by regulating the expression of cathepsin G, vascular endothelial growth factor (VEGF), and monocyte chemotactic protein (MCP)-1 and promotes immune evasion and ECM degradation (Wilson and others 2010; Zu and others 2012). Breast cancer tumor cells overexpress bcl-2 and sFas to ensure their outgrowth and survival, but this coincides with the activation of regulatory mechanisms, such as increased IL-8, TNF-α, LPO, and NO, which attempt to halt tumor cell growth by inducing apoptosis. Ultimately, an imbalance in these mechanisms results in tumor progression, because IL-8, TNF-α, and NO are also angiogenic stimulators (Hamed and others 2012; Kamel and others 2012).

Breast cancer tissues express high concentrations of IL-8 compared with normal tissue (Snoussi and others 2006), which correlates with angiogenesis (Zuccari and others 2012). IL-8 that is secreted by tumor cells enhances endothelial cell proliferation, survival, and MMP production (Hamed and others 2012). In contrast, IL-24, a member of the IL-10 family, suppresses tumor vascularization (Xie and others 2008; Hsu and others 2012).

Chronic inflammation can also lead to angiogenesis, because tumor-infiltrating lymphocytes secrete copious amounts of proinflammatory cytokines, such as IL-6, IL-1α, IL-1β, tumor necrosis factor-α, and oncostatin M, which are believed to upregulate COX-2, which, in turn, increases VEGF expression in tumor cells, promoting angiogenesis (Angelo and Kurzrock 2007). Inflammatory events can also lead to breast cancer metastasis. Further, hypoxic tumor conditions induce COX-2 expression, which activates hypoxia-inducible factor-1α (HIF-1α), a transcription factor that activates angiogenesis-promoting genes, such as *vegf* and *cox-2* (Jung and others 2003; Angelo and Kurzrock 2007) (Fig. 3).

Inflammatory breast cancer exhibits a higher expression of proangiogenic molecules, such as angiopoietin-1, VEGF, and VEGF receptors than noninflammatory breast cancer (Van der Auwera and others 2004; Angelo and Kurzrock 2007).

Cytokines and Breast Cancer Metastasis

Metastasis of breast cancer, such as tumorigenesis and tumor progression, has many mechanisms. Some cytokines in breast cancer, such as TGF-β and IL-6, can promote tumor metastasis through the EMT (Fig. 1), a process that is characterized by reduced expression of E-cadherin and upregulation of markers, such as vimentin and N-cadherin (Culig 2011). CAFs mediate the EMT, producing high amounts of TGF-β (Yilmaz and Christofori 2009; Patel and others 2012).

TAMs are also linked to metastasis, secreting tumor cell migration-stimulating factors, such as CXCL12, IL-6, and TNF (Allavena and others 2008). Macrophage recruitment might be necessary for the progression of breast tumors to a metastatic state, as suggested by studies on a polyoma middle T oncogene (PyMT) mouse model of mammary cancer (Baumgarten and Frasor 2012). Moreover, TAMs might contribute to tumor progression, because TAMs produce estrogen and as conditioned media from TAM cultures stimulate ER-positive breast cancer cells growth (Fig. 2) (Mor and others 1998; Baumgarten and Frasor 2012).

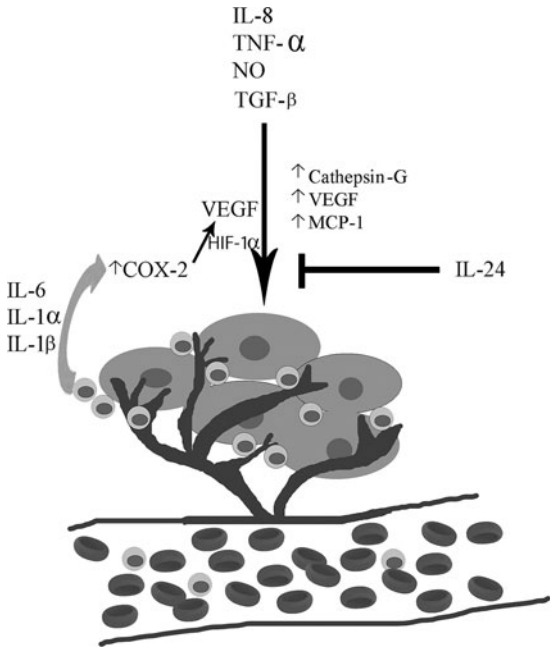

FIG. 3. Cytokines involved in angiogenesis. The inflammatory infiltrate that is usually found in breast tumors produce IL-6, IL-1α, and IL-1β, which upregulate COX-2, which, in turn, increases VEGF expression in tumor cells promoting angiogenesis. IL-8, TNF-α, TGF-β, and NO, produced by tumor cells, are angiogenic stimulators. TGF-β regulates the expression of cathepsin-G, VEGF, and MCP-1, promoting extracellular matrix degradation and angiogenesis. IL-24 suppresses tumor vascularization.

Colony-stimulating factor 1 (CSF-1) might mediate the recruitment of macrophages to breast tumors (Lin and others 2001). The proto-oncogene *c-fms* encodes the only known receptor (CSF-1R) for CSF-1 (Sherr and others 1985; Dai and others 2002). The expression of CSF-1 and its receptor in neoplastic epithelial breast cancer cells correlates well with a poor prognosis and is predictive of ipsilateral recurrence (Scholl and others 1994; Maher and others 1998; Kluger and others 2004). CSF-1 promotes metastasis, stimulates angiogenesis, and participates in a paracrine loop with EGF to spur tumor cell invasion in mouse models (Lin and others 2001; Aharinejad and others 2002; Aharinejad and others 2004; Wyckoff and others 2004).

Breast cancer cell lines consistently express CSF-1 and CSF-1R, which sustains the proliferation in SKBR3 and MDAMB468 breast cancer cells through ERK1/2 activation, stimulating c-Jun and upregulating c-myc and cyclin D1. CSF-1R is not overexpressed or amplified in breast cancer cells compared with human monocytes, suggesting that the oncogenic potential of CSF-1R is attributed to its coexpression with CSF-1 (Morandi and others 2011).

TNF promotes tumor cell invasion, as evidenced in *in vitro* experiments, upregulating several genes that are associated with proliferation, invasion, and metastasis (Yin and others 2009; Baumgarten and Frasor 2012). IL-1 also effects the migration and metastasis of ER-positive cancer cells (Wang and others 2005; Franco-Barraza and others 2010), altering their morphology to assume more of a fibroblast-like appearance and reorganizing the actin cytoskeleton, increasing motility and MMP-9 activity (Duffy and others 2000;

Baumgarten and Frasor 2012). Greater IL-8 expression in breast cancer patients correlates with metastasis (Simeone and others 2007).

IL-19 induces the migration of breast cancer cells, such as Hs578T and 4T1, by upregulating CXCR4, MMP-2, MMP-9, TGF-β, IL-1β, and IL-6—factors that are involved in tumor progression and metastasis. Overexpression of IL-19 in 67NR cells, which usually have low endogenous IL-19 levels, and MCF-7 cells stimulates their proliferation and migration, enabling them to form larger tumors and metastastic micronodules in the lung on injection into mice (Hsing and others 2012). IL-20 *in vitro* upregulates MMP-9, MMP-12, cathepsin K, and cathepsin G and enhances the proliferation and migration of breast cancer cells. IL-20 is highly expressed in breast cancer bone metastases (Hsu and others 2012).

MSC-derived monocyte chemotactic protein-1 (MCP-1/CCL2) and IL-17B promote breast cancer cell migration (Molloy and others 2009; Goldstein and others 2010; De Luca and others 2012). MSCs are a source of factors, such as VEGF and IL-6, that, in addition to promoting angiogenesis, induce breast cancer cell migration and invasion, (Beckermann and others 2008; De Luca and others 2011; De Luca and others 2012). VEGF stimulates the invasion of breast cancer cells by activating MAPK and PI3K/AKT signaling (Price and others 2001).

Hypoxia, characterized by abnormally low levels of oxygen in cells, is a feature of most solid tumors, including breast cancer. This condition orchestrates a series of effects principally regulated by the family of HIFs. HIFs, when translocated to the nucleus in response to low oxygen, induce the expression of a series of factors in cells related to proliferation and survival, metabolism, invasion and metastasis, angiogenesis, pH regulation, and maintenance of stem cells. Between these factors, several cytokines can be found: for example, *TGF-α, Igf-2,* and *Igf-Bp2* (Favaro and others, 2011). In the case of breast cancer, hypoxic conditions induce cytokine and growth factor secretion from MSCs, such as TGF-β1, TGF-β2, and TGF-β3, which affects the growth, motility, and invasiveness of breast cancer cells (Hung and others 2012a, 2012b). Evenmore, TGF-β and hypoxia (through HIF-1α) in parallel drive tumor bone metastases in breast cancer by the regulation of a common set of genes (*CTGF, OPN, MMP-1, IL-6,* and *IL-8,* among others) and additively increment the expression of prometastasic factors VEGF and CXCR4 (Dunn and others, 2009). TGF-β induces the invasiveness of noncarcinogenic epithelial MCF-10A1 (M1) cells and RAS-transformed M1-derived MCF-10AneoT (M2) cells in spheroid assays (Naber and others 2011). Further, levels of TGF-β1 and TGF receptor and cell invasiveness correlate inversely with junctional adhesion molecule-A (JAM-A) expression in breast cancer cell lines (Arteaga and others 1988; Koli and Arteaga 1997; Naik and others 2008). Downregulation of JAM-A due to TGF-β1 involves the TGF-β/Smad and TGF-β/p54 JNK pathways, inducing breast cancer cell invasion (Wang and Lui 2012).

TGF-β1 mediates an EMT-like process, which, in turn, can induce cancer stem cell-like properties in HMLE cells. HMLER cells, which are human primary normal mammary epithelial cells that have been immortalized using hTERT and SV40 large T antigen and H-Ras to render them tumorigenic (Elenbaas and others 2001; Mani and others 2008), potentially lead to early dissemination of breast cancer cells, which can sometimes survive at sites of dissemination and may outgrow after a long latency of years (Podsypanina and others 2008; Sabe 2011).

Inflammatory breast cancer has greater metastatic potential than noninflammatory breast cancer (Van der Auwera and others 2004; Angelo and Kurzrock 2007).

Cytokines, Immunosuppression, and Evasion in Breast Cancer

Breast cancer cells have mechanisms that enable them to grow and progress. As discussed, cytokines are important mediators of tumor growth and metastasis, some of which also help the tumor evade immune responses and benefit from them. For instance, TGF-β binds to MDSCs, causing them to suppress natural killer (NK) cells. TGF-β also influences regulatory T-cell activity (Yoshimura and others 2010) through a neuropilin-1 (Nrp1)-mediated mechanism and supports breast cancer growth (Glinka and Prud'homme 2008; Zu and others 2012).

IL-10 has not only been implicated in the immunosuppression in breast cancer, but it is also involved in the antitumor response. IL-10 is a potent anti-inflammatory cytokine that inhibits gene expression, cytokine synthesis by T cells and macrophages, and their antigen presentation. IL-10 suppresses the production of IL-1α, IL-1β, TNF-α, IL-6, IL-8, IL-12, IL-18, granulocyte–macrophage colony-stimulating factor (GM-CSF), MIP-1α, RANTES, leukemia-inhibiting factor, and itself (Moore and others 1993; Hamidullah and others 2011).

TAMs are a significant source of IL-10 in the tumor microenvironment. TAMs have an immunosuppressive phenotype, characterized by the release of IL-10, likely in response to S1P from dying cancer cells through a complex signaling network that requires S1PR/src-dependent trafficking of TRKA to the plasma membrane in primary human macrophages, on which autocrine NGF induces PI3K/AKT signaling (Weigert and others 2007).

Tumor-associated plasmocytoid dendritic cells (TApDCs) affect immune tolerance through tumor-associated regulatory T-cell expansion and differentiation of IL-10-secreting T cells. The selective suppression of IFN-α production gives TApDC the unique ability to sustain $FoxP3^+$ Treg expansion, contributing to immune tolerance by the tumor and poor clinical outcomes (Sisirak and others 2012).

Moreover, tumor cell lines which are cultured *in vitro* express IL-10, suggesting that IL-10 establishes an immunesuppressive tumor microenvironment. However, overexpression of IL-10 in tumor cells that have been transplanted in mice causes tumors to be rejected, implicating $CD8^+$ T cells, NK cells, or IL-10 (Zheng and others 1996; Moore and others 2001; Mumm and others 2011).

Cytokines as Prognostic Factors in Breast Cancer

In addition to their effects on tumor progression, the levels of several cytokines have been correlated with tumor stage, survival, and malignancy, rendering them potential prognostic factors.

High levels of TGF-β have been linked to worse survival in breast cancer patients, and plasma TGF-β levels might be

predictive of local and distant metastasis (Grau and others 2008; Bierie and others 2009; Ivanović and others 2009; Yu and others 2010; Zu and others 2012). Loss of Wnt5a, which is directly regulated by TGF-β, is associated with early relapse of invasive breast cancer, increased metastasis, and poor survival in breast cancer patients (Serra and others 2011). Higher circulating levels of TGF-β2 correlate with nonpremenopausal status, infiltrating ductal carcinoma, higher histological grade, presence of vascular permeation and lymphocytic infiltration, and longer relapse-free survival, making them likely to be used as a marker of favorable prognosis (Dave and others 2011).

Reduced and increased expression of TGF-β3 in breast tumor biopsies is associated with a poor and a good prognosis, respectively (van de Vijver and others 2002). Similarly, lower *TGF-β3* mRNA levels correlate with higher tumor grade (van de Vijver and others 2002; Miller and others 2005; Ivshina and others 2006; Sotiriou and others 2006; Desmedt and others 2007). Conversely, higher circulating levels of TGF-β3 and TGF-β3-receptor complexes are linked to the presence of lymph node metastases (Laverty and others 2009). Further, TGF-β3 is central to the parity-induced protection against breast cancer, which occurs in women who give birth before the age of 24 years. Parity induces chronic upregulation of TGF-β3 (D'Cruz and others 2002; Laverty and others 2009).

IL-1 family members have prognostic value in breast cancer. IL-1 is expressed particularly in ER-negative breast tumors. Levels of IL-1 in the serum and tumor correlate with invasiveness and a poor prognosis (Goldberg and Schwertfeger 2010). Elevated levels of IL-1β are associated with invasiveness and aggressiveness of breast cancer and higher tumor grade (Jin and others 1997; Chavey and others 2007). High IL-1 receptor antagonist (IL-1ra) levels and low levels of IL-1 at the tumor site are linked to a good prognosis in breast cancer—in the healthy population, individuals with low serum IL-1ra concentrations have higher levels of adiponectin (Rafiq and others 2007); these correlate with increased expression of ERs (Pantschenko and others 2003). Low serum levels of adiponectin are a risk factor for the development of breast cancer in postmenopausal women (Miyoshi and others 2003; Hou and others 2007; Perrier and others 2009).

Moreover, serum IL-6 levels are significantly higher in breast cancer patients than in healthy women, and increased IL-6 levels correlate with poorer survival and diminished response to endocrine therapy in patients with metastatic breast cancer (Zhang and Adachi 1999; Knüpfer and Preiss 2007; Culig 2011; Baumgarten and Frasor 2012; Liu and others 2012a). Plasma from benign breast tumor patients have significantly elevated levels of IL-6 compared with normal controls (Nariţa and others 2011). Patients with high IL-6 concentrations have worse responses to chemotherapy and hormone therapy (Zhang and Adachi 1999). IL-6 levels are lower in breast cancer patients who respond better to therapy (Zhang and Adachi 1999; Guo and others 2012). Nevertheless, IL-6 levels have been reported as positive and negative predictors in breast cancer (Knüpfer and Preiss 2007).

IL-19 expression in breast cancer tissue is associated with a higher mitotic rate, advanced tumor stage, metastasis, and worse disease-specific and metastasis-free survival (Hsing and others 2012), implicating it in progression of the tumor.

In breast cancer, IL-8 is associated with lymph node-positive status, higher stage, and lack of hormone receptors (Zuccari and others 2012). Serum IL-8 has been linked to accelerated clinical progression, greater tumor load, and the presence of lymph node and liver metastases (Benoy and others 2004; Culig 2011). Patients with HER-2/neu + tumors have increased serum IL-8 levels versus those with HER-2/neu- tumors (Vazquez-Martin and others 2007). In contrast, patients with local recurrence or metastases have lower IL-8 levels (Zuccari and others 2012). Plasma IL-8 levels are higher in stage III and IV breast cancer patients compared with stage I and II (Hamed and others 2012).

Circulating TNF levels correlate with higher tumor stage and lymph node metastasis (Sheen-Chen and others 1997). TNF levels are greater in invasive breast cancer tissue than in benign tissue (Miles and others 1994; Baumgarten and Frasor 2012). IL-13 levels show a similar correlation of TNF levels and clinicopathological characteristics in breast cancer patients (Srabovic and others 2011). Higher TNF-α-expressing populations correlate with increasing tumor grade and node involvement (Kamel and others 2012). Similarly, TNF-α plasma levels are elevated in stage II, II, and IV breast cancer patients versus those with stage I and healthy controls (Hamed and others 2012).

IL-10 concentration is frequently higher in the serum of breast cancer patients compared with normal subjects. Elevated IL-10 might inhibit tumor growth by suppressing IL-6 production, based on the inverse correlation between IL-6 and IL-10 levels in cancer patients (Kozłowski and others 2003). IL-10 is overexpressed in ER-negative versus ER-positive breast tumors (Chavey and others 2007). A correlation between IL-10 level and clinical stage has also been reported (Merendino and others 1996)—metastatic disease is associated with higher IL-10 levels than nonmetastatic disease, which might contribute to impaired immunosurveillance, favoring tumor development.

IL-20 is associated with advanced tumor stage, greater tumor metastasis, poor clinical outcome, higher mitotic rate, and worse survival (Hsu and others 2012). Elevated IL-23 levels in breast cancer patients correlate with shorter overall survival (Gangemi and others 2012).

In contrast, higher circulating soluble IL-2R levels appear to be a favorable prognostic indicator (Nicolini and others 2006; Gangemi and others 2012) (Table 1).

Cytokines and Quality of Life in Breast Cancer Patients

Quality of life is a significant issue in breast cancer patients and survival. Patients experience pain, sleep disturbances, and fatigue, even after treatment has ended. More than 54% of patients develop moderate to severe pain during the treatment trajectory (van den Beuken-van Everdingen and others 2007; Starkweather and others 2013).

Increasing evidence suggests that modulation of immune activation through a greater secretion of proinflammatory cytokines accelerates the development of distressing symptoms in women with breast cancer (Lyon and others 2008; Reyes-Gibby and others 2008; Starkweather and others 2013).

Increased levels of IL-1 and IL-6 are associated with pain and sleep disturbances in breast cancer survivors (Collado-Hidalgo and others 2006; Starkweather and others 2013). A significant rise in plasma IL-1ra is also linked to post-treatment

Table 1. Cytokines' Utility for Breast Cancer Prognosis and Survival

Cytokine	*Location*	*Levels*	*Prognosis*	*Impact on survival*	*References*
TGF-β	Tumor	↑	Is predictive of local and distant metastases. Loss of Wnt5a, regulated by TGF-β, is associated with early relapse and metastases	Worse survival	Grau and others (2008); Ivanović and others (2009); Yu and others (2010); Zu and others (2012)
TGF-β2	Serum, tumor	↑	Favorable: In circulation, higher levels correlate with later relapse. *TGF-β2* mRNA expression correlates with early relapse in advanced stage than in early stage of breast cancer Unfavorable: Higher transcript levels in tissue is a marker of unfavorable prognosis	Longer relapse-free survival. mRNA levels correlate with increased survival in early breast cancer than in advanced stage	Dave and others (2011)
TGF-β3	Tumor	↑↓	Reduced expression correlates with poor prognosis and higher tumor grade, while increased expression is indicative of good prognosis	Not determined	Desmedt and others, (2007); Ivshina and others (2006); Miller and others (2005); Sotiriou and others (2006); van de Vijver and others (2002)
TGF-β3	Serum	↑	Higher TGF-β3- TGF-β3 receptor complexes are linked to lymph node metastases: poor prognosis Nonetheless, uprelugulation of this cytokine is involved in the parity-induced protection against breast cancer in women who gave birth before the age of 24 years	Not determined	D'Cruz and others (2002); Laverty and others (2009)
IL-1 and IL-1B	Serum, tumor	↑	High in tumor and circulation: poor prognosis, more aggressive and invasive tumor. High IL-1ra and low IL-1 at tumor site: good prognosis	Not determined	Chavey and others (2007); Goldberg and Schwertfeger (2010); Jin and others (1997); Rafiq and others (2007)
IL-6	Serum	↑	Poor prognosis: diminished response to endocrine therapy and to chemotherapy Lower levels correlate with better response to therapy: good prognosis	Worse survival	Baumgarten and Frasor (2012); Culig, (2011); Knüpfer and Preiss (2007); Liu and others (2012); Zhang and Adachi (1999)
IL-19	Tumor	↑	Associated with higher mitotic rate, advanced tumor stage, and metastasis: poor prognosis	Worse metastasis-free survival	Hsing and others (2012)
IL-8	Tumor	↓	Low levels are associated with lymph node-positive status, lack of hormone receptors, accelerated clinical progression, and metastasis: poor prognosis.	Increased risk of death	Culig (2011); Zuccari and others (2012)
TNF and IL-13	Serum	↑	Higher levels correlate with higher tumor stage and lymph node metastasis	Not determined	Sheen-Chen and others (1997); Srabovic and others (2011)
IL-10	Serum	↑	May inhibit tumor growth by suppressing IL-6. However, higher levels of IL-10 correlate with metastatic disease: poor prognosis	Not determined	Kozłowski and others (2003); Merendino and others (1996)
IL-20	Tumor	↑	Associated with advanced tumor stage, metastasis, and poor clinical outcome: poor prognosis	Worse survival	Hsu and others (2012)
CSF-1	Tumor	↑	Expression in tumor correlates with ipsilateral recurrence of breast cancer: poor prognosis	Not determined	Maher and others (1998); Scholl and others (1994); Kluger and others (2004)
soluble IL-2R	Serum	↑	Good prognosis	Not determined	Gangemi and others (2012); Nicolini an others (2006)
IL-1B	Tumor	↑	Correlates with invasiveness, aggressiveness, and higher tumor grade: poor prognosis	Not determined	Chavey and others (2007); Jin and others (1997)

Different cytokines are listed in this table along with their prognostic values in breast cancer. The location in which each cytokine was evaluated is also indicated as well as their impact on survival.

fatigue in breast cancer survivors (Bower and others 2002; Perrier and others 2009). Similarly, IL-17, IL-13, and CRP levels are higher in women who experience pain, greater pain interference, depression, and sleep disturbances (Starkweather and others 2013). Further, VEGF and soluble intercellular adhesion molecule-1 (sICAM-1) levels are associated with fatigue in patients who are undergoing chemotherapy (Mills and others 2005), as are elevated levels of IL-1RA, soluble tumor necrosis factor receptor type II (sTNF-RII), neopterin, and soluble IL-6 receptor, which have been correlated with fatigue in breast cancer patient survivors at 5 years after diagnosis (Karayiannakis and others 2001; Bower and others 2002; Collado-Hidalgo and others 2006; Berger and others 2012).

CRF might be the pathway at which several systems converge, including the central nervous system, immunoregulatory pathways, and the neuromusculoskeletal and cardiopulmonary systems. The physiological and immunological systems, which control inflammatory pathways, might be involved in CRF (Harvey and others 2009). Moreover, sleep problems have been associated with CRF, depression, and poorer overall quality of life (Lis and others 2008; Schultz and others 2011; Berger and others 2012). Sleep regulatory functions can also be affected by cytokines, such as IL-1, IL-6, and TNF-α (Vgontzas and others 1997; Opp 2005; Raison and others 2010; Liu and others 2012b).

Cytokines and Breast Cancer Treatment

Based on their function in breast cancer development, cytokines are attractive targets for new treatments. Further, cytokines can potentiate or impair the efficacy of current breast cancer therapies. For instance, in the presence of IL-1, the selective ER modulator (SERM) 4-hydroxytamoxifen (4-OHT) activates, rather than represses, the transcription of ER target genes. IL-1 displaces an inhibitory complex that contains NCoR from ER target gene promoters, inhibiting their response to 4-OHT (Zhu and others 2006). Thus, IL-1 might regulate ER activity and responsiveness to endocrine therapy (Baumgarten and Frasor 2012).

TNF-α promotes chemotherapeutic resistance in MCF-7 cells through upregulation of ABCG2, an ATP-binding cassette transporter that effluxes chemotherapeutic drugs from cancer cells (Krishnamurthy and Schuetz 2006; Mosaffa and others 2009; Pradhan and others 2010; Baumgarten and Frasor 2012; Malekshah and others 2012). TGF-β signaling also affects the resistance of breast cancer cells to DNA-damaging chemotherapeutic agents (Yu and others 2010; Zu and others 2012), and it might also induce immunosuppression and lead to resistance and relapse in breast cancer (Joffroy and others 2010; Zu and others 2012).

IL-6 and IL-8 are also related to multidrug resistance in breast cancer cells. Only breast cancer cells that are sensitive to drugs do not express IL-6, whereas multidrug-resistant breast cancer cells produce high levels of IL-6 (Guo and others 2012). Neutralization of IL-6 and IL-8 with antibodies significantly enhances the sensitivity of resistant MCF-7 cells to drugs, and their overexpression increases the resistance of MCF-7/Sensitive cells to chemotherapeutic drugs. Further, IL-6 and IL-8 can be induced by chemotherapeutic drugs (Shi and others 2012), which can lead to multidrug-resistant cells.

IL-6 can mediate trastuzumab (anti-HER2/neu mAb) resistance in breast cancer cells by activating an IL-6-dependent inflammatory feedback loop that causes the expansion of CSCs. IL-6 might induce the CSC phenotype in non-CSC cells. Long-term trastuzumab treatment generates highly enriched CSCs that have an EMT phenotype, secreting over 100 times more IL6 than parental cells. Blocking this IL-6 loop with an antibody against IL-6 receptor interrupts this inflammatory feedback mechanism and decreases the CSC population, resulting in lower tumor growth and metastasis (Korkaya and others 2012).

As therapeutic agents, GM-CSF and granulocyte colony-stimulating factor (G-CSF) have been evaluated with regard to their value in preventing or reducing the incidence of febrile neutropenia (FN) due to high-dose intensive cytotoxic chemotherapy. FN often requires hospitalization and intravenous antibiotics. Peter and others (2013) performed a meta-analysis of several clinical trials and demonstrated that that CSF is beneficial in the prevention of NF. However, CSF can cause adverse events, such as bone pain and injection-site reactions. No conclusions could be drawn from this meta-analysis, as the studies had a few patients, varying definitions, and unclear measurements of trial outcomes.

IFNs, IL-2, and IL-12, cytokines that induce hormone sensitivity and stimulate cellular immunity, have also been used to treat advanced breast cancer (Swain 1991; Fehniger and others 2002; Hsing and others 2012). Although treatment with IL-2 or IL-6 alone, IL-2 combined with IFN-α, G-CSF or trastuzumab, and IL-12 and trastuzumab is well tolerated (Nicolini and others 2006), they had no clear antitumor effect or have improvement beyond each individual treatment. Clinical outcome was also not improved (Nicolini and Carpi 2008). Treatment with IFNs (IFN-α, IFN-β, and IFN-γ), alone or in combination, had no immunological effects or significantly improved clinical outcomes compared with conventional treatment (Nicolini and Carpi 2008).

TGF-β is a notable target, based on its tumor-supportive function in late-stage tumors. Several approaches for the blocking of TGF-β signaling have been developed, including antibodies. The 1D11 antibody, which blocks all 3 isoforms of TGF-β, inhibits 4T1 growth and metastasis. In contrast, blockade of TGF-β in MDAMB-231 cells, which, unlike 4T1 cells, express low levels of TGF-β, does not affect their growth (Liu and others 2012a). TGF-β blockade normalizes the tumor vasculature, improves chemotherapy delivery into tumors, and decreases angiogenic gene expression, including VEGF and IL-8 (Liu and others 2012a). Further, TGF-β blockade normalizes the interstitial matrix by reducing collagen density, improving the transport of nanoparticles from the perivascular space into deeper areas of the tumor, and enhancing the treatment of breast cancer patients (Liu and others 2012a).

1D11 prevents bone metastasis in animal models, significantly reducing tumor burden in the bone and osteolytic lesion areas. Tumor-bearing mice which have been treated with 1D11 double their bone volume, suggesting that anti-TGF-β can be used to treat bone loss in breast cancer (Biswas and others 2011). Moreover, inhibition of TGF-β signaling in human bone marrow MSCs blocks their differentiation into CAFs, induced by the tumor microenvironment, and the consequent protumor effects, improving the safety of MSC-based therapies in cancer patients (Shangguan and others 2012).

Normalization of abnormal vessels in mammary tumors can also be achieved by blocking vascular endothelial growth factor receptor-2 with DC101, an anti-VEGF-receptor-2, improving the delivery of smaller nanoparticles (diameter, 12 nm) while hindering that of larger nanoparticles (diameter, 125 nm) (Chauhan and others 2012), which can be valuable in directing specific drugs to the tumor.

TGF-β signaling can be blocked with other approaches, such as translational inhibition by antisense oligonucleotides, small-molecule inhibitors, and peptide aptamers to Samd proteins (Kelly and Morris 2010; Zu and others 2012). GC1008 and 2G7 are 2 other high-affinity monoclonal antibodies to TGF-β that can neutralize all 3 isoforms, but they are associated with certain drawbacks, such as side effects, including generalized inflammation, autoimmune reactions, and tumorigenic risk (Kim and others 2001; Cheng and others 2008; Zu and others 2012).

The mAb7E anti-IL-20 also reduces tumor growth, suppresses bone colonization, mitigates osteolysis, and improves bone density in mice that have been injected with breast cancer cells (Hsu and others 2012).

Concluding Remarks

New data on breast cancer immunology from the past several years suggest that many long-established and widely accepted paradigms be revised. We have reviewed data that have led to novel models of the biology and function of breast cancer cells. The cytokines that we have discussed are regulated by molecules which were originally believed to be exclusive to the endocrine system: sex steroids. These findings engendered a new concept of bidirectional communication between the endocrine and immune systems under normal circumstances, not necessarily during disease. Finally, the development of drugs that specifically target cytokines, such as the IL-6/sIL-6R pathway, will be valuable in the treatment of breast cancer, in which immune inflammation has a protagonic function.

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Author Disclosure Statement

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References

Aharinejad S, Abraham D, Paulus P, Abri H, Hofmann M, Grossschmidt K, Hofbauer R. 2002. Colony-stimulating factor-1 antisense treatment suppresses growth of human tumor xenografts in mice. Cancer Res 62(18):5317–5324.

Aharinejad S, Paulus P, Sioud M, Hofmann M, Zins K, Schäfer R, Abraham D. 2004. Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. Cancer Res 64(15):5378–5384.

Allavena P, Sica A, Solinas G, Porta C, Mantovani A. 2008. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol 66(1):1–9.

Allington TM, Schiemann WP. 2011. The Cain and Abl of epithelial-mesenchymal transition and transforming growth factor-β in mammary epithelial cells. Cells Tissues Organs 193(1–2):98–113.

Angelo LS, Kurzrock R. 2007. Vascular endothelial growth factor and its relationship to inflammatory mediators. Clin Cancer Res 13(10):2825–2830.

Apte RN, Krelin Y, Song X, Dotan S, Recih E, Elkabets M, Voronov E. 2006. Effects of micro-environment- and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour-host interactions. Eur J Cancer (Oxford, England: 1990) 42(6):751–759.

Arteaga CL, Tandon AK, Von Hoff DD, Osborne CK. 1988. Transforming growth factor beta: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. Cancer Res 48(14):3898–3904.

Balkwill F. 2009. Tumour necrosis factor and cancer. Nat Rev Cancer 9(5):361–371.

Band AM, Laiho M. 2011. Crosstalk of TGF-β and estrogen receptor signaling in breast cancer. J Mammary Gland Biol Neoplasia 16(2):109–115.

Barone I, Catalano S, Gelsomino L, Marsico S, Giordano C, Panza S, Andò S. 2012. Leptin mediates tumor-stromal interactions that promote the invasive growth of breast cancer cells. Cancer Res 72(6):1416–1427.

Baumgarten SC, Frasor J. 2012. Minireview: inflammation: an instigator of more aggressive estrogen receptor (ER) positive breast cancers. Mol Endocrinol 26(3):360–371.

Beckermann BM, Kallifatidis G, Groth A, Frommhold D, Apel A, Mattern J, Herr I. 2008. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. Br J Cancer 99(4):622–631.

Benoy IH, Salgado R, Van Dam P, Geboers K, Van Marck E, Scharpé S, Dirix LY. 2004. Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. Clin Cancer Res 10(21):7157–7162.

Berger AM, Gerber LH, Mayer DK. 2012. Cancer-related fatigue: implications for breast cancer survivors. Cancer 118(8 Suppl):2261–2269.

Bierie B, Chung CH, Parker JS, Stover DG, Cheng N, Chytil A, Moses HL. 2009. Abrogation of TGF-beta signaling enhances chemokine production and correlates with prognosis in human breast cancer. J Clin Invest 119(6):1571–1582.

Biswas S, Nyman JS, Alvarez J, Chakrabarti A, Ayres A, Sterling J, Mundy GR. 2011. Anti-transforming growth factor ß antibody treatment rescues bone loss and prevents breast cancer metastasis to bone. PloS One 6(11):e27090.

Booth BW, Smith GH. 2007. Roles of transforming growth factor-alpha in mammary development and disease. Growth Factors 25(4):227–235.

Bower JE, Ganz PA, Aziz N, Fahey JL. 2002. Fatigue and proinflammatory cytokine activity in breast cancer survivors. Psychosom Med 64(4):604–611.

Bruunsgaard H, Pedersen M, Pedersen BK. 2001. Aging and proinflammatory cytokines. Curr Opin Hematol 8(3):131–136.

Byers T, Sedjo RL. 2011. Does intentional weight loss reduce cancer risk? Diabetes Obes Metab 13(12):1063–1072.

Cai D. 2009. NFkappaB-mediated metabolic inflammation in peripheral tissues versus central nervous system. Cell Cycle 8(16):2542–2548.

Caldefie-Chézet F, Damez M, de Latour M, Konska G, Mishellani F, Fusillier C, Vasson M-P. 2005. Leptin: a proliferative factor for breast cancer? Study on human ductal carcinoma. Biochem Biophys Res Commun 334(3):737–741.

Caldefie-Chezet F, Poulin A, Tridon A, Sion B, Vasson MP. 2001. Leptin: a potential regulator of polymorphonuclear neutrophil bactericidal action? J Leukoc Biol 69(3):414–418.

Caldefie-Chezet F, Poulin A, Vasson MP. 2003. Leptin regulates functional capacities of polymorphonuclear neutrophils. Free Radic Res 37(8):809–814.

Casey TM, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Plaut K. 2008. Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-beta 1) increase invasion rate of tumor cells: a population study. Breast Cancer Res Treat 110(1):39–49.

Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML, Andò S. 2003. Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. J Biol Chem 278(31): 28668–28676.

Chauhan VP, Stylianopoulos T, Martin JD, Popović Z, Chen O, Kamoun WS, Jain RK. 2012. Normalization of tumour blood vessels improves the delivery of nanomedicines in a size-dependent manner. Nat Nanotechnol 7(6):383–388.

Chavey C, Bibeau F, Gourgou-Bourgade S, Burlinchon S, Boissière F, Laune D, Lazennec G. 2007. Oestrogen receptor negative breast cancers exhibit high cytokine content. Breast Cancer Res 9(1):R15.

Cheng N, Chytil A, Shyr Y, Joly A, Moses HL. 2008. Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. Mol Cancer Res 6(10):1521–1533.

Cleary MP, Grossmann ME. 2009. Minireview: Obesity and breast cancer: the estrogen connection. Endocrinology 150(6):2537–2542.

Collado-Hidalgo A, Bower JE, Ganz PA, Cole SW, Irwin MR. 2006. Inflammatory biomarkers for persistent fatigue in breast cancer survivors. Clin Cancer Res 12(9):2759–2766.

Connelly L, Barham W, Onishko HM, Sherrill T, Chodosh LA, Blackwell TS, Yull FE. 2011. Inhibition of NF-kappa B activity in mammary epithelium increases tumor latency and decreases tumor burden. Oncogene 30(12):1402–1412.

Coussens LM, Werb Z. 2002. Inflammation and cancer. Nature 420(6917):860–867.

Culig Z. 2011. Cytokine disbalance in common human cancers. Biochim Biophys Acta 1813(2):308–314.

D'Cruz CM, Moody SE, Master SR, Hartman JL, Keiper EA, Imielinski MB, Chodosh LA. 2002. Persistent parity-induced changes in growth factors, TGF-beta3, and differentiation in the rodent mammary gland. Mol Endocrinol 16(9):2034–2051.

D'Esposito V, Passaretti F, Hammarstedt A, Liguoro D, Terracciano D, Molea G, Formisano P. 2012. Adipocyte-released insulin-like growth factor-1 is regulated by glucose and fatty acids and controls breast cancer cell growth *in vitro*. Diabetologia 55(10):2811–2822.

Dai X-M, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, Stanley ER. 2002. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood 99(1):111–120.

Dave H, Trivedi S, Shah M, Shukla S. 2011. Transforming growth factor beta 2: a predictive marker for breast cancer. Indian J Exp Biol 49(11):879–887.

De Luca A, Gallo M, Aldinucci D, Ribatti D, Lamura L, D'Alessio A, Normanno N. 2011. Role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells. J Cell Physiol 226(8):2131–2138.

De Luca A, Lamura L, Gallo M, Maffia V, Normanno N. 2012. Mesenchymal stem cell-derived interleukin-6 and vascular endothelial growth factor promote breast cancer cell migration. J Cell Biochem 113(11):3363–3370.

Desmedt C, Piette F, Loi S, Wang Y, Lallemand F, Haibe-Kains B, Sotiriou C. 2007. Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. Clin Cancer Res 13(11):3207–3214.

Dieudonne M-N, Bussiere M, Dos Santos E, Leneveu M-C, Giudicelli Y, Pecquery R. 2006. Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. Biochem Biophys Res Commun 345(1):271–279.

Dinarello CA. 1996. Biologic basis for interleukin-1 in disease. Blood 87(6):2095–2147.

Donato NJ, Klostergaard J. 2004. Distinct stress and cell destruction pathways are engaged by TNF and ceramide during apoptosis of MCF-7 cells. Exp Cell Res 294(2):523–533.

Donovan J, Slingerland J. 2000. Transforming growth factor-beta and breast cancer: Cell cycle arrest by transforming growth factor-beta and its disruption in cancer. Breast Cancer Res 2(2):116–124.

Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N. 2000. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. Breast Cancer Res 2(4):252–257.

Dunn LK, Mohammad KS, Fournier PGJ, McKenna CR, Davis HW, Niewolna M, Guise TA. 2009. Hypoxia and TGF-beta drive breast cancer bone metastases through parallel signaling pathways in tumor cells and the bone microenvironment. PloS One 4(9):e6896.

Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Weinberg RA. 2001. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev 15(1):50–65.

Ernst M, Putoczki TL. 2012. Stat3: linking inflammation to (gastrointestinal) tumourigenesis. Clin Exp Pharmacol Physiol 39(8):711–718.

Ewan KBR, Oketch-Rabah HA, Ravani SA, Shyamala G, Moses HL, Barcellos-Hoff MH. 2005. Proliferation of estrogen receptor-alpha-positive mammary epithelial cells is restrained by transforming growth factor-beta1 in adult mice. Am J Pathol 167(2):409–417.

Faggioni R, Fantuzzi G, Fuller J, Dinarello CA, Feingold KR, Grunfeld C. 1998. IL-1 beta mediates leptin induction during inflammation. Am J Physiol 274(1 Pt 2):R204–R208.

Favaro E, Lord S, Harris AL, Buffa FM. 2011. Gene expression and hypoxia in breast cancer. Genome Med 3(8):55.

Fehniger TA, Cooper MA, Caligiuri MA. 2002. Interleukin-2 and interleukin-15: immunotherapy for cancer. Cytokine Growth Factor Rev 13(2):169–183.

Feng X-H, Liang Y-Y, Liang M, Zhai W, Lin X. 2002. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(Ink4B). Mol Cell 9(1):133–143.

Figueroa JD, Flanders KC, Garcia-Closas M, Anderson WF, Yang XR, Matsuno RK, Sherman ME. 2010. Expression of TGF-beta signaling factors in invasive breast cancers:

relationships with age at diagnosis and tumor characteristics. Breast Cancer Res Treat 121(3):727–735.

Franco-Barraza J, Valdivia-Silva JE, Zamudio-Meza H, Castillo A, García-Zepeda EA, Benítez-Bribiesca L, Meza I. 2010. Actin cytoskeleton participation in the onset of IL-1beta induction of an invasive mesenchymal-like phenotype in epithelial MCF-7 cells. Arch Med Res 41(3):170–181.

Fujita Y, Murakami M, Ogawa Y, Masuzaki H, Tanaka M, Ozaki S, Mimori T. 2002. Leptin inhibits stress-induced apoptosis of T lymphocytes. Clin Exp Immunol 128(1):21–26.

Gangemi S, Minciullo P, Adamo B, Franchina T, Ricciardi GRR, Ferraro M, Adamo V. 2012. Clinical significance of circulating interleukin-23 as a prognostic factor in breast cancer patients. J Cell Biochem 113(6):2122–2125.

Glasgow E, Mishra L. 2008. Transforming growth factor-beta signaling and ubiquitinators in cancer. Endocr Relat Cancer 15(1):59–72.

Glinka Y, Prud'homme, G. J. 2008. Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity. J Leukoc Biol 84(1):302–310.

Goldberg JE, Schwertfeger KL. 2010. Proinflammatory cytokines in breast cancer: mechanisms of action and potential targets for therapeutics. Curr Drug Targets 11(9):1133–1146.

Goldstein RH, Reagan MR, Anderson K, Kaplan DL, Rosenblatt M. 2010. Human bone marrow-derived MSCs can home to orthotopic breast cancer tumors and promote bone metastasis. Cancer Res 70(24):10044–10050.

Grau AM, Wen W, Ramroopsingh DS, Gao Y-T, Zi J, Cai Q, Zheng W. 2008. Circulating transforming growth factor-beta-1 and breast cancer prognosis: results from the Shanghai Breast Cancer Study. Breast Cancer Res Treat 112(2):335–341.

Grivennikov SI, Greten FR, Karin M. 2010. Immunity, inflammation, and cancer. Cell 140(6):883–899.

Guo Y, Xu F, Lu T, Duan Z, Zhang Z. 2012. Interleukin-6 signaling pathway in targeted therapy for cancer. Cancer Treat Rev 38(7):904–910.

Hamed EA, Zakhary MM, Maximous DW. 2012. Apoptosis, angiogenesis, inflammation, and oxidative stress: basic interactions in patients with early and metastatic breast cancer. J Cancer Res Clin Oncol 138(6):999–1009.

Hamidullah, Changkija B, Konwar R. 2011. Role of interleukin-10 in breast cancer. Breast Cancer Res Treat 133(1):11–21.

Harvey SB, Wessely S, Kuh D, Hotopf M. 2009. The relationship between fatigue and psychiatric disorders: evidence for the concept of neurasthenia. J Psychosom Res 66(5):445–454.

He D, Li H, Yusuf N, Elmets CA, Li J, Mountz JD, Xu H. 2010. IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells. J Immunol 184(5):2281–2288.

Heldin C-H, Landström M, Moustakas A. 2009. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Curr Opin Cell Biol 21(2):166–176.

Honma S, Shimodaira K, Shimizu Y, Tsuchiya N, Saito H, Yanaihara T, Okai T. 2002. The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells. Endocr J 49(3):371–377.

Hou W-K, Xu Y-X, Yu T, Zhang L, Zhang W-W, Fu C-L, Chen L. 2007. Adipocytokines and breast cancer risk. Chin Med J 120(18):1592–1596.

Hsing C-H, Cheng H-C, Hsu Y-H, Chan C-H, Yeh C-H, Li C-F, Chang M-S. 2012. Upregulated IL-19 in breast cancer promotes tumor progression and affects clinical outcome. Clin Cancer Res 18(3):713–725.

Hsu Y-H, Hsing C-H, Li C-F, Chan C-H, Chang M-C, Yan J-J, Chang M-S. 2012. Anti-IL-20 monoclonal antibody suppresses breast cancer progression and bone osteolysis in murine models. J Immunol 188(4):1981–1991.

Hu X, Juneja SC, Maihle NJ, Cleary MP. 2002. Leptin—a growth factor in normal and malignant breast cells and for normal mammary gland development. J Natl Cancer Inst 94(22):1704–1711.

Humphreys RC, Hennighausen L. 2000. Transforming growth factor alpha and mouse models of human breast cancer. Oncogene 19(8):1085–1091.

Hung S-P, Ho JH, Shih Y-R. V, Lo T, Lee OK. 2012a. Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. J Orthop Res 30(2): 260–266.

Hung S-P, Yang M-H, Tseng K-F, Lee OK. 2012b. Hypoxia-induced secretion of TGF-beta 1 in mesenchymal stem cell promotes breast cancer cell progression. Cell Transplant 22(10):1869–1882.

Iguchi M, Aiba S, Yoshino Y, Tagami H. 2001. Human follicular papilla cells carry out nonadipose tissue production of leptin. J Invest Dermatol 117(6):1349–1356.

Iliopoulos D, Hirsch HA, Struhl K. 2009. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell 139(4):693–706.

Iliopoulos D, Hirsch HA, Wang G, Struhl K. 2011. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proc Natl Acad Sci U S A 108(4):1397–1402.

Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. 2010. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. Mol Cell 39(4):493–506.

Imamura T, Hikita A, Inoue Y. 2012. The roles of TGF-β signaling in carcinogenesis and breast cancer metastasis. Breast Cancer 19(2):118–124.

Inman GJ. 2011. Switching TGFβ from a tumor suppressor to a tumor promoter. Curr Opin Genet Dev 21(1):93–99.

Irahara N, Miyoshi Y, Taguchi T, Tamaki Y, Noguchi S. 2006. Quantitative analysis of aromatase mRNA expression derived from various promoters (I.4, I.3, PII and I.7) and its association with expression of TNF-alpha, IL-6 and COX-2 mRNAs in human breast cancer. Int J of Cancer 118(8): 1915–1921.

Ivanović V, Dedović-Tanić N, Milovanović Z, Lukić S, Nikolić S, Baltić V, Dimitrijević B. 2009. Quantification of transforming growth factor beta 1 levels in metastatic axillary lymph node tissue extracts from breast cancer patients: a new specimen source. Anal Quant Cytol Histol 31(5):288–295.

Ivshina AV, George J, Senko O, Mow B, Putti TC, Smeds J, Miller LD. 2006. Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. Cancer Res 66(21):10292–10301.

Jain P, Alahari SK. 2011. Breast cancer stem cells: a new challenge for breast cancer treatment. Front Biosci 16:1824–1832.

Jardé T, Caldefie-Chézet F, Damez M, Mishellany F, Penault-Llorca F, Guillot J, Vasson MP. 2008. Leptin and leptin receptor involvement in cancer development: a study on human primary breast carcinoma. Oncol Rep 19(4):905–911.

Jin L, Yuan RQ, Fuchs A, Yao Y, Joseph A, Schwall R, Rosen EM. 1997. Expression of interleukin-1beta in human breast carcinoma. Cancer 80(3):421–434.

Joffroy CM, Buck MB, Stope MB, Popp SL, Pfizenmaier K, Knabbe C. 2010. Antiestrogens induce transforming growth

factor beta-mediated immunosuppression in breast cancer. Cancer Res 70(4):1314–1322.
Joshi A, Cao D. 2010. TGF-beta signaling, tumor microenvironment and tumor progression: the butterfly effect. Front Biosci 15:180–194.
Juárez P, Guise TA. 2010. Tgf-Beta pathway as a therapeutic target in bone metastases. Curr Pharm Des 16(11):1301–1312.
Jung Y-J, Isaacs JS, Lee S, Trepel J, Neckers L. 2003. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J 17(14):2115–2117.
Kalluri R, Weinberg RA. 2009. The basics of epithelial-mesenchymal transition. J Clin Invest 119(6):1420–1428.
Kamel M, Shouman S, El-Merzebany M, Kilic G, Veenstra T, Saeed M, Salama S. 2012. Effect of tumour necrosis factor-alpha on estrogen metabolic pathways in breast cancer cells. J Cancer 3:310–321.
Kang JH, Lee YY, Yu BY, Yang B-S, Cho K-H, Yoon DK, Roh YK. 2005. Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. Arch Pharm Res 28(11):1263–1269.
Karayiannakis AJ, Syrigos KN, Polychronidis A, Pitiakoudis M, Bounovas A, Simopoulos K. 2001. Serum levels of tumor necrosis factor-alpha and nutritional status in pancreatic cancer patients. Anticancer Res 21(2B):1355–1358.
Karin M, Greten FR. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5(10):749–759.
Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Weinberg RA. 2007. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449(7162):557–563.
Kelly RJ, Morris JC. 2010. Transforming growth factor-beta: a target for cancer therapy. J Immunotoxicol 7(1):15–26.
Kim KY, Jeong SY, Won J, Ryu PD, Nam MJ. 2001. Induction of angiogenesis by expression of soluble type II transforming growth factor-beta receptor in mouse hepatoma. J Biol Chem 276(42):38781–38786.
Kluger HM, Dolled-Filhart M, Rodov S, Kacinski BM, Camp RL, Rimm DL. 2004. Macrophage colony-stimulating factor-1 receptor expression is associated with poor outcome in breast cancer by large cohort tissue microarray analysis. Clin Cancer Res 10(1 Pt 1):173–177.
Knüpfer H, Preiss R. 2007. Significance of interleukin-6 (IL-6) in breast cancer (review). Breast Cancer Res Treat 102(2):129–135.
Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, Orimo A. 2010. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A 107(46):20009–20014.
Koli KM, Arteaga CL. 1997. Predominant cytosolic localization of type II transforming growth factor beta receptors in human breast carcinoma cells. Cancer Res 57(5):970–977.
Korkaya H, Kim G-I, Davis A, Malik F, Henry NL, Ithimakin S, Wicha MS. 2012. Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. Mol Cell 47(4):570–584.
Korkaya H, Liu S, Wicha MS. 2011. Review series Breast cancer stem cells, cytokine networks, and the tumor microenvironment. J Clin Invest 121(10):3804–3809.
Kozłowski L, Zakrzewska I, Tokajuk P, Wojtukiewicz MZ. 2003. Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. Rocz Akad Med Białymst 48:82–84.
Krishnamurthy P, Schuetz JD. 2006. Role of ABCG2/BCRP in biology and medicine. Ann Rev Pharmacol Toxicol 46:381–410.
Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Oft M. 2006. IL-23 promotes tumour incidence and growth. Nature 442(7101):461–465.
Laud K, Gourdou I, Pessemesse L, Peyrat JP, Djiane J. 2002. Identification of leptin receptors in human breast cancer: functional activity in the T47-D breast cancer cell line. Mol Cell Endocrinol 188(1–2):219–226.
Laverty HG, Wakefield LM, Occleston NL, O'Kane S, Ferguson MWJ. 2009. TGF-beta3 and cancer: a review. Cytokine Growth Factor Rev 20(4):305–317.
Leek RD, Landers R, Fox SB, Ng F, Harris AL, Lewis CE. 1998. Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. Br J Cancer 77(12):2246–2251.
Lewis AM, Varghese S, Xu H, Alexander HR. 2006. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J Transl Med 4:48.
Lin EY, Nguyen AV, Russell RG, Pollard JW. 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 193(6):727–740.
Lin S, Yang J, Elkahloun AG, Bandyopadhyay A, Wang L, Cornell JE, Sun L-Z. 2012. Attenuation of TGF-β signaling suppresses premature senescence in a p21-dependent manner and promotes oncogenic Ras-mediated metastatic transformation in human mammary epithelial cells. Mol Biol Cell 23(8):1569–1581.
Lis CG, Gupta D, Grutsch JF. 2008. The relationship between insomnia and patient satisfaction with quality of life in cancer. Support Care Cancer 16(3):261–266.
Liu J, Liao S, Diop-frimpong B, Chen W, Goel S, Naxerova K, Xu L. 2012a. TGF-β blockade improves the distribution and efficacy of therapeutics in breast carcinoma by normalizing the tumor stroma. Proc Natl Acad Sci U S A 109(41):16618–16623.
Liu L, Mills PJ, Rissling M, Fiorentino L, Natarajan L, Dimsdale JE, Ancoli-Israel S. 2012b. Fatigue and sleep quality are associated with changes in inflammatory markers in breast cancer patients undergoing chemotherapy. Brain Behav Immun 26(5):706–713.
Lyon DE, McCain NL, Walter J, Schubert C. 2008. Cytokine comparisons between women with breast cancer and women with a negative breast biopsy. Nurs Res 57(1):51–58.
Maher MG, Sapi E, Turner B, Gumbs A, Perrotta PL, Carter D, Haffty BG. 1998. Prognostic significance of colony-stimulating factor receptor expression in ipsilateral breast cancer recurrence. Clin Cancer Res 4(8):1851–1856.
Malekshah OM, Lage H, Bahrami AR, Afshari JT, Behravan J. 2012. PXR and NF-κB correlate with the inducing effects of IL-1β and TNF-α on ABCG2 expression in breast cancer cell lines. Eur J Pharm Sci 47(2):474–480.
Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, Weinberg RA. 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133(4):704–715.
Martín-Romero C, Santos-Alvarez J, Goberna R, Sánchez-Margalet V. 2000. Human leptin enhances activation and proliferation of human circulating T lymphocytes. Cell Immunol 199(1):15–24.
Massagué J. 2008. TGFbeta in Cancer. Cell 134(2):215–230.

Merendino RA, Arena A, Capozza AB, Chillemi S, Mesiti M. 1996. Serum levels of interleukin-10 in patients affected by breast cancer. Immunol Lett 53(1):59–60.

Meulmeester E, Ten Dijke P. 2011. The dynamic roles of TGF-β in cancer. J Pathol 223(2):205–218.

Miles DW, Happerfield LC, Naylor MS, Bobrow LG, Rubens RD, Balkwill FR. 1994. Expression of tumour necrosis factor (TNF alpha) and its receptors in benign and malignant breast tissue. Int J Cancer 56(6):777–782.

Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Bergh J. 2005. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A 102(38):13550–13555.

Miller LJ, Kurtzman SH, Anderson K, Wang Y, Stankus M, Renna M, Kreutzer DL. 2000. Interleukin-1 family expression in human breast cancer: interleukin-1 receptor antagonist. Cancer Invest 18(4):293–302.

Mills PJ, Parker B, Dimsdale JE, Sadler GR, Ancoli-Israel S. 2005. The relationship between fatigue and quality of life and inflammation during anthracycline-based chemotherapy in breast cancer. Biol Psychol 69(1):85–96.

Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y, Noguchi S. 2003. Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res 9(15):5699–5704.

Molloy AP, Martin FT, Dwyer RM, Griffin TP, Murphy M, Barry FP, Kerin MJ. 2009. Mesenchymal stem cell secretion of chemokines during differentiation into osteoblasts, and their potential role in mediating interactions with breast cancer cells. Int J Cancer 124(2):326–332.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. Ann Rev Immunol 19:683–765.

Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. 1993. Interleukin-10. Ann Rev Immunol 11:165–190.

Mor G, Yue W, Santen RJ, Gutierrez L, Eliza M, Berstein LM, Naftolin F. 1998. Macrophages, estrogen and the microenvironment of breast cancer. J Steroid Biochem Mol Biol 67(5–6):403–411.

Morandi A, Barbetti V, Riverso M, Dello Sbarba P, Rovida E. 2011. The colony-stimulating factor-1 (CSF-1) receptor sustains ERK1/2 activation and proliferation in breast cancer cell lines. PloS One 6(11):e27450.

Mosaffa F, Lage H, Afshari JT, Behravan J. 2009. Interleukin-1 beta and tumor necrosis factor-alpha increase ABCG2 expression in MCF-7 breast carcinoma cell line and its mitoxantrone-resistant derivative, MCF-7/MX. Inflamm Res 58(10):669–676.

Mumm JB, Emmerich J, Zhang X, Chan I, Wu L, Mauze S, Oft M. 2011. IL-10 elicits IFNγ-dependent tumor immune surveillance. Cancer Cell 20(6):781–796.

Naber HPH, Wiercinska E, Ten Dijke P, van Laar T. 2011. Spheroid assay to measure TGF-β-induced invasion. J Vis Exp (57):1–7.

Naik MU, Naik TU, Suckow AT, Duncan MK, Naik UP. 2008. Attenuation of junctional adhesion molecule-A is a contributing factor for breast cancer cell invasion. Cancer Res 68(7):2194–2203.

Nariţa D, Seclaman E, Ursoniu S, Ilina R, Cireap N, Anghel A. 2011. Expression of CCL18 and interleukin-6 in the plasma of breast cancer patients as compared with benign tumor patients and healthy controls. Rom J Morphol Embryol 52(4):1261–1267.

Nicolini A, Carpi A. 2008. Immune manipulation of advanced breast cancer: an interpretative model of the relationship between immune system and tumor cell biology. Med Res Rev 29(3):436–471.

Nicolini A, Carpi A, Rossi G. 2006. Cytokines in breast cancer. Cytokine Growth Factor Rev 17(5):325–337.

Novitskiy SV, Pickup MW, Gorska AE, Owens P, Chytil A, Aakre M, Moses HL. 2011. TGF-β receptor II loss promotes mammary carcinoma progression by Th17 dependent mechanisms. Cancer Discov 1(5):430–441.

O'brien SN, Welter BH, Price TM. 1999. Presence of leptin in breast cell lines and breast tumors. Biochem Biophys Res Commun 259(3):695–698.

Opp MR. 2005. Cytokines and sleep. Sleep Med Rev 9(5):355–364.

Orimo A, Weinberg RA. 2006. Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 5(15):1597–1601.

Ostman A, Augsten M. 2009. Cancer-associated fibroblasts and tumor growth—bystanders turning into key players. Curr Opin Genet Dev 19(1):67–73.

Otero M, Lago R, Gómez R, Lago F, Gómez-Reino JJ, Gualillo O. 2006. Leptin: a metabolic hormone that functions like a proinflammatory adipokine. Drug News Perspect 19(1): 21–26.

Ouchi N, Parker JL, Lugus JJ, Walsh K. 2011. Adipokines in inflammation and metabolic disease. Nat Rev Immunol 11(2):85–97.

Pantschenko AG, Pushkar I, Anderson KH, Wang Y, Miller LJ, Kurtzman SH, Kreutzer DL. 2003. The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression. Int J Oncol 23(2):269–284.

Patel LR, Camacho DF, Shiozawa Y, Pienta KJ. 2012. Mechanisms of cancer cell metastasis to the bone: a multistep process. Future Oncol 7(11):1285–1297.

Perk J, Iavarone A, Benezra R. 2005. Id family of helix-loop-helix proteins in cancer. Nat Rev Cancer 5(8):603–614.

Perrier S, Caldefie-Chézet F, Vasson M-P. 2009. IL-1 family in breast cancer: potential interplay with leptin and other adipocytokines. FEBS Lett 583(2):259–265.

Peter R, Stefania M, Ping LJ, Marcel Z, Josef B, Markus H. 2013. Primary prophylactic colony-stimulating factors for the prevention of chemotherapy-induced febrile neutropenia in breast cancer patients. Cochrane Database Syst Rev 10: CD007913.

Pfeilschifter J, Köditz R, Pfohl M, Schatz H. 2002. Changes in proinflammatory cytokine activity after menopause. Endocrine Rev 23(1):90–119.

Podsypanina K, Du Y-CN, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. 2008. Seeding and propagation of untransformed mouse mammary cells in the lung. Science 321(5897):1841–1844.

Pradhan M, Bembinster LA, Baumgarten SC, Frasor J. 2010. Proinflammatory cytokines enhance estrogen-dependent expression of the multidrug transporter gene ABCG2 through estrogen receptor and NF{kappa}B cooperativity at adjacent response elements. J Biol Chem 285(41):31100–31106.

Price DJ, Miralem T, Jiang S, Steinberg R, Avraham H. 2001. Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. Cell Growth Differ 12(3):129–135.

Rafiq S, Stevens K, Hurst AJ, Murray A, Henley W, Weedon MN, Frayling TM. 2007. Common genetic variation in the gene encoding interleukin-1-receptor antagonist (IL-1RA) is

associated with altered circulating IL-1RA levels. Genes Immun 8(4):344–351.
Raison CL, Rye DB, Woolwine BJ, Vogt GJ, Bautista BM, Spivey JR, Miller AH. 2010. Chronic interferon-alpha administration disrupts sleep continuity and depth in patients with hepatitis C: association with fatigue, motor slowing, and increased evening cortisol. Biol Psychiatry 68(10):942–949.
Reichlin S, Chen G, Nicolson M. 2000. Blood to brain transfer of leptin in normal and interleukin-1beta-treated male rats. Endocrinology 141(6):1951–1954.
Reyes-Gibby CC, Wu X, Spitz M, Kurzrock R, Fisch M, Bruera E, Shete S. 2008. Molecular epidemiology, cancer-related symptoms, and cytokines pathway. Lancet Oncol 9(8):777–785.
Sabe H. 2011. Cancer early dissemination: cancerous epithelial-mesenchymal transdifferentiation and transforming growth factor β signalling. J Biochem 149(6):633–639.
Sasser AK, Sullivan NJ, Studebaker AW, Hendey LF, Axel AE, Hall BM. 2007. Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer. FASEB J 21(13): 3763–3770.
Scholl SM, Pallud C, Beuvon F, Hacene K, Stanley ER, Rohrschneider L, Lidereau R. 1994. Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. J Natl Cancer Inst 86(2):120–126.
Schultz SL, Dalton SO, Christensen J, Carlsen K, Ross L, Johansen C. 2011. Factors correlated with fatigue in breast cancer survivors undergoing a rehabilitation course, Denmark, 2002–2005. Psycho Oncol 20(4):352–360.
Serra R, Easter SL, Jiang W, Baxley SE. 2011. Wnt5a as an effector of TGFβ in mammary development and cancer. J Mammary Gland Biol Neoplasia 16(2):157–167.
Shangguan L, Ti X, Krause U, Hai B, Zhao Y, Yang Z, Liu F. 2012. Inhibition of TGF-β/Smad signaling by BAMBI blocks differentiation of human mesenchymal stem cells to carcinoma-associated fibroblasts and abolishes their protumor effects. Stem Cells 30(12):2810–2819.
Sheen-Chen SM, Chen WJ, Eng HL, Chou FF. 1997. Serum concentration of tumor necrosis factor in patients with breast cancer. Breast Cancer Res Treat 43(3):211–215.
Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. 1985. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41(3):665–676.
Shi Z, Yang W-M, Chen L-P, Yang D-H, Zhou Q, Zhu J, Huang R-P. 2012. Enhanced chemosensitization in multidrug-resistant human breast cancer cells by inhibition of IL-6 and IL-8 production. Breast Cancer Res Treat 135(3): 737–747.
Simeone A-M, Nieves-Alicea R, McMurtry VC, Colella S, Krahe R, Tari AM. 2007. Cyclooxygenase-2 uses the protein kinase C/interleukin-8/urokinase-type plasminogen activator pathway to increase the invasiveness of breast cancer cells. Int J Oncol 30(4):785–792.
Singer CF, Kronsteiner N, Hudelist G, Marton E, Walter I, Kubista M, Kubista E. 2003. Interleukin 1 system and sex steroid receptor expression in human breast cancer: interleukin 1alpha protein secretion is correlated with malignant phenotype. Clin Cancer Res 9(13):4877–4883.
Singh G, Singh SK, König A, Reutlinger K, Nye MD, Adhikary T, Ellenrieder V. 2010. Sequential activation of NFAT and c-Myc transcription factors mediates the TGF-beta switch from a suppressor to a promoter of cancer cell proliferation. J Biol Chem 285(35):27241–27250.
Sisirak V, Faget J, Gobert M, Goutagny N, Vey N, Treilleux I, Bendriss-Vermare N. 2012. Impaired IFN-α production by plasmacytoid dendritic cells favors regulatory T-cell expansion that may contribute to breast cancer progression. Cancer Res 72(20):5188–5197.
Snoussi K, Mahfoudh W, Bouaouina N, Ahmed SB, Helal AN, Chouchane L. 2006. Genetic variation in IL-8 associated with increased risk and poor prognosis of breast carcinoma. Hum Immunol 67(1–2):13–21.
Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, Delorenzi M. 2006. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst 98(4):262–272.
Srabovic N, Mujagic Z, Mujanovic-mustedanagic J, Muminovic Z, Softic A, Begic L. 2011. Interleukin 13 expression in the primary breast cancer tumor tissue. Biochem Medica 21(2): 131–138.
Starkweather AR, Lyon DE, Schubert CM. 2013. Pain and inflammation in women with early-stage breast cancer prior to induction of chemotherapy. Biol Res Nurs 15(2):234–241.
Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavolari S, Hall BM. 2008. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. Cancer Res 68(21):9087–9095.
Sullivan NJ, Sasser AK, Axel AE, Vesuna F, Raman V, Ramirez N, Hall BM. 2009. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene 28(33):2940–2947.
Swain SL. 1991. Lymphokines and the immune response: the central role of interleukin-2. Curr Opin Immunol 3(3):304–310.
Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Mani SA. 2010. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci U S A 107(35):15449–15454.
Tian M, Schiemann WP. 2010. PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-beta signaling during mammary tumorigenesis. FASEB J 24(4):1105–1116.
Tilg H, Moschen AR. 2006. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 6(10):772–783.
Van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AAM, Voskuil DW, Bernards R. 2002. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347(25):1999–2009.
Van den Beuken-van Everdingen MHJ, de Rijke JM, Kessels AG, Schouten HC, van Kleef M, Patijn J. 2007. High prevalence of pain in patients with cancer in a large population-based study in The Netherlands. Pain 132(3):312–320.
Van der Auwera I, Van Laere SJ, Van den Eynden GG, Benoy I, van Dam P, Colpaert CG, Dirix LY. 2004. Increased angiogenesis and lymphangiogenesis in inflammatory versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. Clin Cancer Res 10(23):7965–7971.
Vazquez-Martin A, Colomer R, Menendez JA. 2007. Protein array technology to detect HER2 (erbB-2)-induced ''cytokine signature'' in breast cancer. Eur J Cancer 43(7):1117–1124.
Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. Immunity 24(2):179–189.

Vgontzas AN, Papanicolaou DA, Bixler EO, Kales A, Tyson K, Chrousos GP. 1997. Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. J Clin Endocrinol Metab 82(5):1313–1316.

Vincent T, Neve EPA, Johnson JR, Kukalev A, Rojo F, Albanell J, Fuxe J. 2009. A SNAIL1–SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial–mesenchymal transition. Nat Cell Biol 11(8):943–950.

Voulgari A, Pintzas A. 2009. Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. Biochim Biophys Acta 1796(2):75–90.

Walter M, Liang S, Ghosh S, Hornsby PJ, Li R. 2009. Interleukin 6 secreted from adipose stromal cells promotes migration and invasion of breast cancer cells. Oncogene 28(30): 2745–2755.

Wang F, Liu H, Liu S, Tang S, Yang L, Feng G. 2005. SHP-2 promoting migration and metastasis of MCF-7 with loss of E-cadherin, dephosphorylation of FAK and secretion of MMP-9 induced by IL-1beta *in vivo* and *in vitro*. Breast Cancer Res Treat 89(1):5–14.

Wang Y, Lui W-Y. 2012. Transforming growth factor-β1 attenuates junctional adhesion molecule-A and contributes to breast cancer cell invasion. Eur J Cancer 48(18):3475–3487.

Weigert A, Tzieply N, von Knethen A, Johann AM, Schmidt H, Geisslinger G, Brüne B. 2007. Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. Mol Biol Cell 18(10):3810–3819.

Wendt MK, Allington TM, Schiemann WP. 2009. Mechanisms of the epithelial-mesenchymal transition by TGF-beta. Future Oncol 5(8):1145–1168.

Wilson TJ, Nannuru KC, Futakuchi M, Singh RK. 2010. Cathepsin G-mediated enhanced TGF-beta signaling promotes angiogenesis via upregulation of VEGF and MCP-1. Cancer Lett 288(2):162–169.

Wolf JS, Chen Z, Dong G, Sunwoo JB, Bancroft CC, Capo DE, Van Waes C. 2001. IL (interleukin)-1alpha promotes nuclear factor-kappaB and AP-1-induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. Clin Cancer Res 7(6):1812–1820.

Wu M-H, Chou Y-C, Chou W-Y, Hsu G-C, Chu C-H, Yu C-P, Sun C-A. 2009. Circulating levels of leptin, adiposity and breast cancer risk. Br J Cancer 100(4):578–582.

Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Condeelis J. 2004. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 64(19):7022–7029.

Xie G, Yao Q, Liu Y, Du S, Liu A, Guo Z, Yuan Y. 2012. IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. Int J Oncol 40(4):1171–1179.

Xie Y, Sheng W, Xiang J, Ye Z, Zhu Y, Chen X, Yang J. 2008. Recombinant human IL-24 suppresses lung carcinoma cell growth via induction of cell apoptosis and inhibition of tumor angiogenesis. Cancer Biother Radiopharm 23(3):310–320.

Xu J, Lamouille S, Derynck R. 2009. TGF-beta-induced epithelial to mesenchymal transition. Cell Res 19(2):156–172.

Yilmaz M, Christofori G. 2009. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 28(1–2):15–33.

Yin X, Wolford CC, Chang Y-S, McConoughey SJ, Ramsey SA, Aderem A, Hai T. 2010. ATF3, an adaptive-response gene, enhances TGF{beta} signaling and cancer-initiating cell features in breast cancer cells. J Cell Sci 123(Pt 20):3558–3565.

Yin Y, Chen X, Shu Y. 2009. Gene expression of the invasive phenotype of TNF-alpha-treated MCF-7 cells. Biomed Pharmacother 63(6):421–428.

Yoshimura A, Wakabayashi Y, Mori T. 2010. Cellular and molecular basis for the regulation of inflammation by TGF-beta. J Biochem 147(6):781–792.

Yu Y, Wang Y, Ren X, Tsuyada A, Li A, Liu LJ, Wang SE. 2010. Context-dependent bidirectional regulation of the MutS homolog 2 by transforming growth factor β contributes to chemoresistance in breast cancer cells. Mol Cancer Res 8(12):1633–1642.

Zhang GJ, Adachi I. 1999. Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. Anticancer Res 19(2B):1427–1432.

Zhao Y, Sun R, You L, Gao C, Tian Z. 2003. Expression of leptin receptors and response to leptin stimulation of human natural killer cell lines. Biochem Biophys Res Commun 300(2):247–252.

Zheng LM, Ojcius DM, Garaud F, Roth C, Maxwell E, Li Z, King I. 1996. Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. J Exp Med 184(2):579–584.

Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Littman DR. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 8(9):967–974.

Zhu P, Baek SH, Bourk EM, Ohgi KA, Garcia-Bassets I, Sanjo H, Rose DW. 2006. Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. Cell 124(3):615–629.

Ziober BL, Willson JK, Hymphrey LE, Childress-Fields K, Brattain MG. 1993. Autocrine transforming growth factor-alpha is associated with progression of transformed properties in human colon cancer cells. J Biol Chem 268(1): 691–698.

Zu X, Zhang Q, Cao R, Liu J, Zhong J, Wen G, Cao D. 2012. Transforming growth factor-β signaling in tumor initiation, progression and therapy in breast cancer: an update. Cell Tissue Res 347(1):73–84.

Zuccari DAPDC, Leonel C, Castro R, Gelaleti GB, Jardim BV, Moscheta MG, Esteves JL. 2012. An immunohistochemical study of interleukin-8 (IL-8) in breast cancer. Acta Histochem 114(6):571–576.

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The Immunoendocrine Network in Breast Cancer

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Abstract. Breast cancer is a disease in which abnormal cell proliferation leads to uncontrolled growth of breast tissue. Breast cancer can start in various areas of the breast, such as the ducts, lobes, and, in some cases, the intervening tissue. For many years, inflammatory infiltrates in tumors have been suggested to reflect the origin of cancer; however, little is known about the function of chronic inflammation in malignant transformation.

Sex hormones are associated with many types of cancer, such as colon, cervical, and especially breast. Estrogen-dependent breast cancer (EDBC) constitutes approximately 50% to 80% of all cases of breast cancer. Furthermore, estrogen-dependency is linked to the initiation of malignancy by promoting the growth and proliferation of mammary cells and it is related to prognosis and treatment. The correlation between sex hormones and breast cancer has been recognized for decades, but the mechanisms of this association remain unknown.

In recent years, a more enriched landscape of this relationship has emerged. Intervention by the immune system in cancer begins with the detection of transformed cells and their proliferation-—not with the defense and effort to restrain an established tumoral mass. In the late 1950s, Burnet introduced the immunosurveillance theory, which proposes that immune system cells detect and attack transformed cells, eliciting an adaptive response that succeeds and eliminates them or fails, leading to the formation of a tumoral mass and cancer onset.

Conversely, sex hormones are important modulators of the immune system. Growing evidence demonstrates a reciprocal relationship between sex steroids and the immune system. Because the innate immune response determines the type of adaptive immunity that develops, hormonal effects on the former can affect adaptive responses. The sex steroids estrogens, progesterone, and testosterone regulate the growth, differentiation, survival, and function of many cell types that mediate homeostasis, immunity, and breast cancer. The presence of sex steroid receptors on immune cells indicates that sex steroids exert their effects by binding to them. Sex steroids and immunity are inextricably linked, and their mutual regulation influences the maintenance of the immune balance.

Understanding the mechanisms of action of sex steroids on immune cells is paramount to developing novel therapies for chronic diseases that are associated to immune dysregulation, such as breast cancer. This chapter describes the risk factors in breast cancer, the hormonal factors that are involved, the immunological response toward cancer, and the effects of sex steroids on immune system cells and their implications for the incidence of breast cancer.

Keywords: Breast cancer, sex steroids, immune response, immunoendocrine network

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INTRODUCTION

The immune and neuroendocrine systems are integrated through a complex network of hormones, neurohormones, cytokines, chemokines, neurotransmitters, and neuropeptides—all of which maintain homeostasis [1, 2]. Two of the principal components of this network are the hypothalamic-pituitary-adrenocortical (HPA) axis [3] and the hypothalamic-pituitary-gonadal axis (HPG) [4]. The relationship between the HPA and HPG axes and the immune system during acute and chronic inflammatory responses due to stress, infection, cancer, and autoimmune responses is well established [5–10].

An important aspect of cellular communication that has emerged from studies of the neuroendocrine and immune system is the redundancy in the chemical messengers that mediate the communication. The identification of many cellular messengers that coordinate the interactions between these homeostatic systems may herald a fundamental shift in our understanding of such significant processes as neurotransmission, neuromodulation, and host immune responses. Thus, there is an extremely complex neuroimmunoendocrine (NIE) network that involves many molecules, including cytokines, neurotransmitters, hormones, and neurohormones, that foresees potent interactions in events generally attributed to the exclusive operation of single systems in response to simple precepts (eg, neurotransmission, reproduction, defense).

The plasticity and multifunctionality of a network, however, carry certain risks. Loss of control in the NIE network might accelerate the development of pathologies in which inflammation is a prominent effector, such as breast cancer; affect the loss of tolerance and autoimmunity; and mediate the compromise in the immune system with age. The interneuronal communication between multiple synaptic, presynaptic, and parasynaptic interactions is further complicated by the intimate participation of cytokines in these processes, which results in a complex network that must be balanced delicately during all ontogenic states of an organism. The mutual regulatory influences between neurotransmitters and the neurotrophic and neuroimmune systems demonstrate that under normal conditions, they work in concert.

Breast cancer is a disease in which abnormal cell proliferation leads to uncontrolled growth of breast tissue. Breast cancer can develop in many areas of the breast, such as the ducts, lobes, and, in some cases, the intervening tissue. The name of the type of cancer is based on its origin. Table 1 summarizes the types of breast cancer by histopathology.

RISK FACTORS OF BREAST CANCER

There are several risk factors for breast cancer, of which sex is the most significant. Other risks factors include genetics, ethnicity, age, and obesity.

Breast cells are immature and highly reactive, even when the breast has fully formed—a condition that remains until a woman carries to the end of her first pregnancy. These immature cells respond primarily to estrogen. 17β-estradiol is classified as a carcinogen by the International Agency on Cancer [11, 12], because natural levels in humans are carcinogenic, as evidenced by the breast cancer risk that is associated with menarche and late menopause [13].

Pregnancy induces breast cells to mature and grow more regularly and decreases the total number of menstrual cycles, lowering estradiol levels. Women who have not had a full-term pregnancy and become pregnant after age 30 years, begin menstrual cycles at an early age, or have had delayed menopause are more likely to develop breast cancer, due to longer exposure to estradiol. Breast-feeding, in contrast, lowers the risk of breast cancer, because milk production causes breast cells to behave normally, decreasing estrogen levels [14]. In addition, obesity increases the risk of developing breast cancer, because adipocytes eliminate produce estradiol [15].

Other endocrine disruptors, defined by the US Environmental Protection Agency as exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, also induce cancer. Such compounds include natural xenoestrogens and chemical agents, such as bisphenol A (BPA), parabens, phthalates, pesticides, hormones for consumption of animal feed, heterocyclic amines (HCAs) that form when meat is grilled, and polycyclic aromatic hydrocarbons (PAHs) that are formed when fat is burned on meat that is grilled [16]. Xenoestrogens have less affinity for estrogen nuclear receptor than estradiol and synergize with endogenous hormones [17], rendering the exposure to xenoestrogens a concern due to the rise in serum levels of steroid hormones.

Bisphenol A is an endocrine disruptor that is ubiquitous in the environment. Exposure to this compound can begin during fetal development. Based on many experimental models, there are many effects of bisphenol A on the development and predisposition to breast cancer in adults [18, 19].

Hormone replacement therapy and treatment with phytohormones or synthetic hormones to reduce the unpleasant effects of menopause also increase the risk

Table 1
Histological classification of breast carcinoma

Type	Subtype	Invasivity	Cell abnormality	Receptors	Other characteristics	Symptoms
Ductal carcinoma	*In situ*	NO	Hyperplasia, some abnormal appearance	–	Confined to ducts	NO
	Tubular	YES	Low malignancy: cells similar to normal and slow growth. Few chromosomal aberrations	Hormone receptors (+), HER2 (–)	Tube-like structures	Swelling, redness, or tenderness in breast. A small lump
	Medullary	YES	Extensive cell abnormality	Hormone receptors (+), HER2 (–)	Lax tumor	Swelling, redness, or tenderness in breast. A small lump
Mucinous carcinoma	–	A few	Usually, no chromosomal aberrations	Hormone receptors (+), HER2 (–)	Cells are floating, scattered on a viscous environment (mucin)	Eventual small lump
Papillary carcinoma	–	YES	Abnormal, fast-growing cells	–	Well-defined edges made of finger-like projections	NO
Lobular carcinoma	*In situ*	NO	Some abnormal cells that remain in the lobe	–	Not considered a true cancer but an indication of possible cancer development	NO
	Infiltrating	YES	Diverse histological features	–	Low or absent expression of E-cadherin or CDH1	Thickening or hardening in the breast that can be felt, rather than a distinct lump
Inflammatory breast cancer	–	Yes	High malignancy, high-grade aneuploidy, very rapid growth and expansion	Hormone receptors (–)	Mutations and overexpression of p53, high levels and abnormal function of E-cadherin	Redness and/or swelling rather than a lump. Heaviness or thickening of breast

of breast cancer. Therapy is riskier when estrogen and progesterone are replaced. Estrogen replacement is risky only when administered for over 10 years [20].

With regard to genetics, a strong history of breast cancer record is associated with mutations in *BRCA 1* and *BRCA 2* (breast cancer genes 1 and 2). The proteins that are encoded by these genes are essential for DNA—BRCA1 and 2 bind and regulate RAD51 to repair cuts in DNA that are caused naturally, on exposure to mutagens, and when chromosomes exchange genetic material during meiosis. By repairing DNA, these proteins maintain stability in the human genome, preventing dangerous rearrangements that can lead to the development of various cancers [21].

Both BRCA genes are tumor suppressor genes. A germinal cell with a mutation in a gene generates daughter cells, all of which will carry the mutation. A healthy allele maintains a gene's function; thus, the development of a carcinoma entails the loss of a normal allele [22].

The expression of these genes is regulated hormonally [23]. They are expressed in epithelial cells during development, increasing during pregnancy and declining after childbirth. In breast cancer cells, BRCA1 and BRCA2 mRNA is upregulated by estrogen [24]. In Mexico, an exploratory study reported that 9% of patients aged 35 years or less with breast cancer diagnostic, had mutations in BRCA1 and BRCA2 genes, wich was consistent with international rates and the types of mutations were similar to those founded in other countries. The types of mutations that were identified were similar to those in other countries [25]. In some breast cancers, mutations develop in other genes but are less common and do not appear to increase the risk compared with abnormalities in BRCA 1 and 2.

Patients with the genetic disease ataxia-telangiectasia carry mutations in the ATM (ataxia telangiectasia mutated) gene, causing them to lose brain cells and muscle control, predispose them to developing cancer, and increase their sensitivity to radiation. ATM is a kinase, an enzyme that phosphorylates other proteins. ATM mediates DNA repair, and its absence decreases a cell's capacity to repair mutations. Abnormalities in this gene are associated with breast cancer [26].

p53 encodes a nuclear phosphoprotein that instructs a cell to synthesize proteins that halt tumor growth. Inheritance of mutant *p53* causes Li-Fraumeni syndrome, a disorder in which patients develop soft tissue cancers (including breast) at an early age [27]. *CHEK2* (cell cycle gene checkpoint kinase 2) is a tumor suppressor that is activated by ATM and phosphorylates various substrates, including Cdc25A, Cdc25C, p53, and BRCA1, leading to cell cycle arrest, apoptosis, and DNA repair. Mutations in this gene increase the likelihood of cancer (including breast cancer) [28].

PTEN (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase) is a phosphatase—a protein that removes a phosphate group from tyrosine, serine, and threonine residues in phosphorylated proteins—that hydrolyzes the phosphate group of carbon 3 of phosphatidylinositol-3,4,5-trisphosphate. PTEN is also a tumor suppressor. Uncontrolled activation of the PI3K pathway accelerates cellular transformation and tumor progression in several tumor types, including brain, breast, ovarian, and renal carcinoma. PTEN dephosphorylates PIP_3, thus regulating PI3K signaling. PTEN has a protein-tyrosine phosphatase domain and a domain with homology to tensin, suggesting that PTEN suppresses tumor cell growth by antagonizing tyrosine protein kinases and regulating the invasion of tumor cells during metastasis and interactions with focal adhesions [29].

CDH1 encodes a cadherin (epithelial cadherin) 1, which lies on the cell surface and maintains cellular cohesion in forming organized tissue. It also transmits chemical signals in the cell that can halt cell division and growth. Abnormal CDH1 methylation occurs at high frequencies in infiltrating breast cancers, in association with a decrease in E-cadherin expression [30].

Breast cancer has a higher incidence in Caucasian woman than the African-Hispanic and Asian populations, but it is more aggressive in African woman (26). These differences in mortality, however, can be influenced by other factors, such as timely care, eating habits, and living conditions [31].

HORMONAL FACTORS IN BREAST CANCER

It has long been suggested that inflammatory infiltrates in tumors reflect the origins of cancer, but little is known about the function of chronic inflammation in malignant transformation. The nature of the inflammatory response is likely responsible for angiogenesis—the Th1 response is considered proinflammatory, driving cell-mediated immunity, whereas Th2 response is immune-regulatory or anti-inflammatory and promotes antibody-mediated immunity [32].

The mammary gland undergoes several stages of differentiation throughout life, which are modulated by

the hormones 17β-estradiol and progesterone during *in utero* modulation, puberty, menstruation, pregnancy, lactation, weaning, and menopause. Moreover, the modern lifestyle has imposed other factors with regard to breast development, differentiation, maturation, and eventual carcinogenesis. The mammary gland comprises 2 cellular compartments: a highly dynamic epithelium that undergoes cycles of proliferation, differentiation, and apoptosis in response to local and endocrine signals and an underlying stroma that contains fibroblasts, endothelial cells, and adipocytes, which constitute the mammary fat pad [33]. The epithelium of the mammary gland is composed of luminal and basal/myoepithelial cells [34].

Breast cancer is caused by the subversion of normal growth regulatory pathways in mammary epithelial cells due to genetic mutations and epigenetic modifications in tumor suppressors, oncogenes, and DNA repair genes. The luminal and basal subtypes of breast cancer are defined by the gene expression patterns that reflect these lineages. Moreover, luminal tumors assume a more differentiated and less aggressive state than their basal counterparts [35, 36].

Estrogens are required to induce complete ductal elongation and branching and the development of lobulo-alveolar end buds [37]. There is substantial experimental, clinical, and epidemiological evidence that suggests that endogenous estrogen levels are a factor of risk of breast cancer; however, the direct involvement of estrogens is debated, because the epidemiological studies have only reported a positive association [38].

Estrogens exert their effects through 2 receptors, alpha and beta (ERα and ERβ). ERα signaling is required in the mammary epithelium for ductal elongation during puberty and stromal invasion, whereas ERβ is widely expressed in the epithelial lining and stroma of normal mammary glands [33]. The function of ERβ is still under investigation, because most studies have focused on ERα.

In breast cancer, ERα is an established prognostic marker and therapeutic target, and ERβ has emerged as significant factor of the response to endocrine treatment—ERβ expression in invasive breast cancer is linked significantly improved survival rates, primarily in postmenopausal women [33]. In normal mammary glands, $ER\alpha^+$ cells do not proliferate, however, when cancer is present, this property changes and they begin to proliferate. Normal breast duct cells rarely divide and are primarily $ER\alpha^-$; approximately 10% of normal ductal cells express ERα. In the early stages of carcinogenesis, ERα is upregulated; nearly 70% of invasive breast carcinomas eventually express this receptor [39].

Nuclear ER expression is important for mammary gland development, but its relevance in estrogen-induced breast cancer is unknown. Some studies have postulated that estrogens cause breast cancer through an ERα-independent mechanism. Further, several environmental compounds, natural and synthetic, can be recognized by ERα and ERβ and might interact with specific residues to generate estrogenic or antiestrogenic responses.

In addition to steroidal estrogens, other some compounds, such as phytoestrogens and xenoestrogens, regulate the development and differentiation of breast. Flavonoids, isoflavonoids, cumestanes, and lignans are phytoestrogens that have greater affinity for ERβ, the isoform that is associated with antitumor properties [40]—the high levels of these compounds in many products of plant origin might explain the notable geographic differences in breast cancer incidence. Similar to steroidal estrogens, phytoestrogens regulate transcription and have rapid effects through extranuclear signaling cascades; thus, phytoestrogens have a wide range of activities, independent of ERs.

Xenoestrogens are present in many synthetic pesticides, plasticizers, detergents, and cosmetics, and some studies suggest that their activity depends on the ERα/ERβ ratio in tissue [40]. Because cancer is considered as aging-associated disease, exposure to compounds that modulate or disrupt endocrine function during critical periods of early development might influence the susceptibility to breast cancer in adulthood [33].

The function of androgens in breast cancer development and progression has not been determined completely; epidemiological studies have reported a positive association between high androgen levels and the risk of cancer [41]. There are 5 principal androgens in premenopausal women: dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, testosterone, and dihydrotestoterone (DHT).

Sex hormones have a significant function in the development and progression of breast cancer, as do ovarian hormones. Most studies support a relationship between higher circulating androgens levels and increased breast cancer risk [42]. In contrast, some models contend that androgens protect the breast from estrogen-induced stimulation, increased proliferation, and cancer development, likely due to competitive blockade from the inhibitory effects of androgens on the mammary epithelium [43]. Epidemiological

evidence suggests a positive association between higher levels of circulating androgens and estrogens and postmenopausal breast cancer; 9 prospective studies of menopausal women reported a greater risk of breast cancer with increasing circulating sex hormone levels (including estradiol, estrone sulfate, androstenedione, DHEA, DHEAS and testosterone) [44]. One prospective study reported no association between progesterone levels and breast cancer risk [45], whereas two other studies noted that the link between hormone levels and breast cancer risk was independent of ER and progesterone status [46, 47].

The direct effects of testosterone and DHT are mediated through their binding to androgen receptor (AR); AR is expressed in approximately 80% of primary breast cancers, similar to ER (70%) and progesterone (60%) [36, 48, 49]. AR expression in breast cancer is associated with a favorable response to endocrine treatment and prognosis, particularly for ER^+ breast cancer, whereas AR expression and survival do not correlate in women with ER^- breast cancers. AR expression is linked to improved clinico-pathological features and improved survival in women with triple-negative breast cancer ($ER^-/PR^-/AR^-$) [41].

Sex hormones are associated with many types of cancer, such as colon [50, 51], cervical [52], and especially breast [53, 54]. Estrogen-dependent breast cancer (EDBC) constitutes approximately 50% to 80% of all cases of breast cancer [55, 56]. Further, estrogen-dependency is associated with the onset of malignancy through the induction of mammary cell growth and proliferation, which allow setting this type of cancer into a particular scenario of prognostic and treatment, where estrogens rule the tumorigenic process [57, 58].

Estrogens, particularly estradiol, stimulate cellular proliferation and differentiation of the breast during normal development. It mediates its effects primarily through 2 intracellular receptors, ERα and ERβ, which form homo- and heterodimers. When estrogen binds to its receptor, conformational changes take place that promote receptor dimerization and the recruitment of coactivators that ultimately induce transcription [59]. As a result, alterations occur in cellular proliferation and the differentiation of reproductive organs, such as breast, uterus, and ovary, and other organs and tissues, such as bone, brain, cardiovascular tissue, and the genitourinary tract. Notably, excessive or sustained exposure of cells to estrogens can lead to chronic proliferation and promote malignant transformation [57]. In addition, estrogen expression in tumoral breast tissue has significant clinical relevance with regard to tumor growth and persistence [60, 61].

The link between sex hormones and breast cancer was made several decades ago, but the mechanisms of this association remain unknown. In recent years, a more enriched landscape of this relationship has emerged. Estradiol, the most biologically active estrogen, is produced by the conversion of androgens by aromatase. Aromatase expression is higher in breast cancer (BC) tissue than in nontumoral, benign breast lesions [61]. Further, the relative risk for BC correlates with age at first pregnancy. A full-term pregnancy before age 30 years is associated with protection from or delay in the development of BC, whereas first pregnancies that occur later increase this risk [62, 63]. In addition, the level of circulating estradiol is often linked to greater risk of BC [13, 47], and a recent report has demonstrated that the expression of many estrogen-responsive genes is enhanced in BC tissue [64]. Abnormally high levels of circulating androstenedione and testosterone are also associated with increased risk of developing BC, because they can be converted into estrogens by aromatase [57, 65]. Thus, prolonged exposure to elevated endogenous estrogens, particularly estradiol, correlates with a greater risk of developing breast cancer.

The significance of ER and PR status in the prognosis and treatment of EDBC has also been analyzed in large worldwide cohorts, of which ER status is the strongest prediction of prognosis versus age, race, tumor stage, and size. Tables 2 and 3 briefly summarize the findings of ER and PR expression in large cohorts of BC patients in the US.

Table 2
Effect of receptor status on outcome of tamoxifen treatment of breast cancer*

	Mortality risk reduction	Reduction in risk of recurrence	Disease-free survival at 5 years
ER (+) PR (+)	46%	53%	82.5%
ER (+) PR (-)	30%	25%	73.8%

*Data from databases of 15871 patients. Values are relative to ER^- and PR^- patients [55].

Table 3
Effect of receptor status on mortality risk in untreated breast cancer patients*

	Elevation in mortality risk (fold)	Elevation in mortality risk by tumor grade of
$ER^- PR^+$	1.5 to 2.1	
$ER^+ PR^-$	1.2 to 1.5	
$ER^- PR^-$	2.6	low lesion: 2.6 to 3.1 high lesion: 2.1 to 2.3

*Data are from 155,175 patients. Values are relative to or compared with ER+ PR+ patients [56].

Abnormally high estrogen levels are linked to carcinogenesis in the breast through 2 biochemical pathways. In the first pathway, excess estradiol modulates the transcription and activity of second messengers that initiate a cascade of altered gene expression that can enhance cellular proliferation and impede apoptosis.

The other pathway entails the metabolism of estradiol to its genotoxic oxidative metabolites. Estradiol can be transformed into 6-alpha hydroxyestrone, which covalently binds to DNA and DNA-binding proteins, or 4-hydroxiestradiol quinone—a metabolite that induces adducts of quinone and DNA oxidative damage—through the catechol estrogen pathway [57, 66, 67]. Ultimately these processes can effect uncontrolled cellular proliferation and malignant transformation (Figure 1).

In postmenopausal women, in whom ovarian production of estradiol has ceased, stromal cells from breast tissue supply estrogen to cancer cells. Two recent studies in mice have implicated estrogens in the support of tumoral repopulation by inducing the selective recruitment of cancer breast stem cells from luminal tissue into the tumor. Notably, most, if not all, mobilized cells are estrogen receptor-negative.

Despite the elevated levels of circulating estradiol in EDBC, genomic and epigenetic alterations occur that can precede the genesis and progression of a malignancy, predisposing one to cancer and disease progression—some of these changes are linked to estradiol production and its metabolism. Many studies have correlated the increased risk of BC to single-nucleotide polymorphisms (SNPs) in steroid 17-alpha-monooxygenascytochrome P-450 17

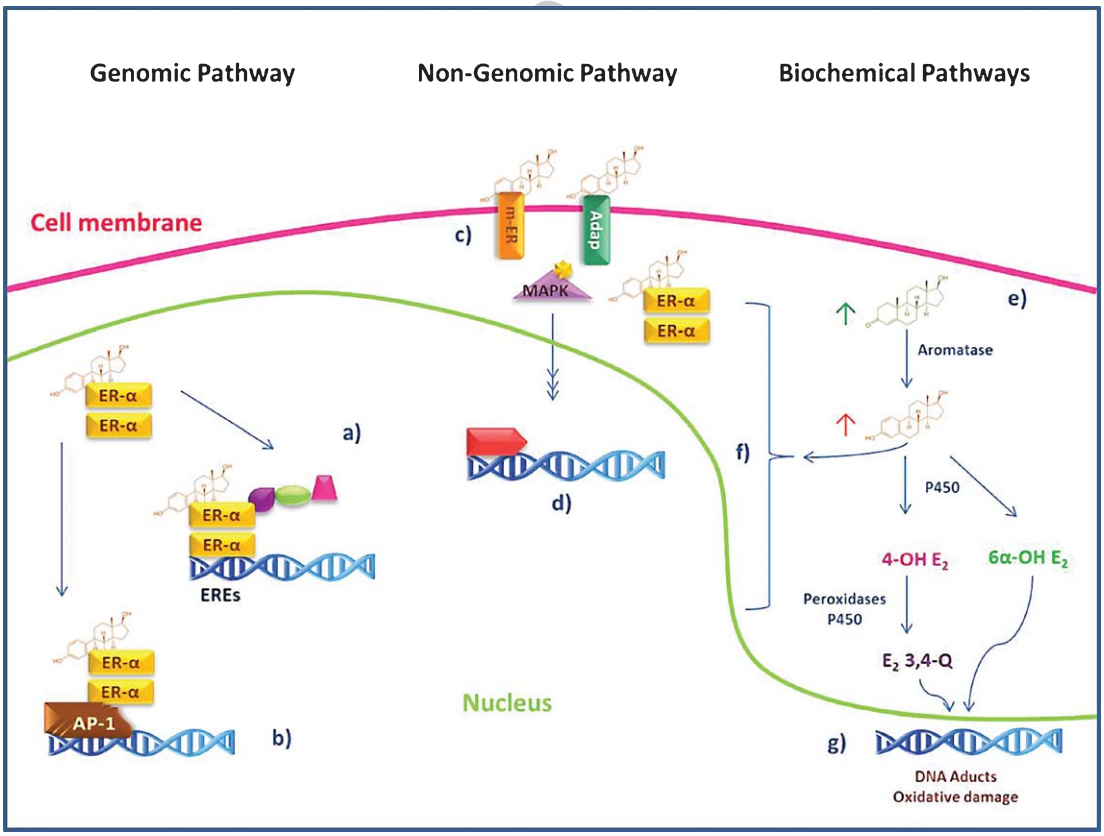

Fig. 1. Hormonal factors in breast cancer. The genomic pathway toward E_2 transcriptional effects begins with the interaction of ERα or ERβ with E_2. The ER-E_2 complex then acts as a a) ligand-activated transcription factor that binds directly to estrogen-responsive elements (EREs) and recruits coactivators to promote transcription or b) coregulator of other transcription factors. In the nongenomic pathway, c) E_2, via adaptor membrane receptors or in ER-E_2 complexes, activates MAP kinases and other transcription factors, initiating signaling cascades that effect d) the transcription of target genes. Two biochemical pathways have been postulated to explain the correlation between high circulating androgen levels and breast cancer. e) Testosterone is converted into E_2 by aromatase, and f) the resulting E_2 functions as described above. g) Estradiol is metabolized by P450 cytochrome, and some of its metabolites, such as 6-alpha hydroxyestradiol and estradiol 3,4-quinone, bind directly to DNA and cause oxidative damage.

(CYP17), which encodes an enzyme that converts pregnenonolone and progesterone into DHEA and androstenedione, which eventually can be reduced to estradiol. The T-to-C SNP of at position 1931 in the promoter of CYP 17 is associated with higher levels of circulating estradiol and progestin [68]. Also, greater frequency of TTTA repeats in the aromatase gene has been linked to higher aromatase activity, although this finding has not been confirmed definitively.

Polymorphisms in other enzymes that metabolize estradiol are also associated with a higher risk of BC, such as CYP1A1, GSTM1, and COMT, which can increase estradiol levels, [69]. In a Mexican population, a significant association between rs1048943 in CYP1A1 (OR = 1.95, C.I. 1.13–3.36) and rs1695 in GSTP1 (OR = 2.39, C.I. 1.24–4.24) and the risk of breast cancer has been demonstrated [70].

Thus, the participation of other factors might be necessary to overcome or potentiate the effects of these SNPs on BC risk. The overexpression of ERα has garnered particular biological and clinical interest, because it is observed during the early stages of breast cancer and is a favorable predictor of prognosis in response to hormonal treatment [71].

The distal section of promoter B of ERα is an enhancer. The expression of ERα wanes as the disease progresses toward more aggressive and malignant stages. Hypermethylation of promoter B is frequently associated with advanced BC and decreased ERα expression. In ERα-positive breast cancer cell lines, the methylation of this region downregulates ERα transcription. Demethylation of promoter B induces the recruitment of the *trans*-acting factor ERBF-1, which is expressed specifically in response to ERα transcription from promoter B. Notably, in ERα-negative breast cancer cell lines, demethylation of promoter B does not upregulate this receptor or ERBF-1 [72].

In addition, ER can be associated with lipid rafts on activation by its ligand, which can promote its interaction with Her-2, an oncogene that is associated with acceleration of breast cancer progression through cellular proliferation and inhibition of apoptosis. Thus, epigenetic alterations, such as DNA methylation; novel linking with oncogenic proteins; and the absence of activating factors can predetermine or alter the hormonal dependence of the tumor and disease outcomes.

The current treatment of EDBC comprises surgical excision of the tumor and sex-hormone-producer and chemotherapy with adjuvant hormone therapy. One type of hormone therapy is based on selective estrogen receptor modulators (SERMs), which are ER antagonists. Tamoxifen is the most effective ERSM against EDBC and is more successful when cancer cells are ER^+ and PR^+. Tamoxifen prevents the binding of the ER ligand, distorting the conformation of the ER and precluding coactivators and factors that are essential for ER-dependent tumor growth from binding.

In addition to hormone therapy, aromatase inhibitors (AIs) have been developed to treat BC and ovarian cancer, against which they are more effective than tamoxifen and have less toxicity. AIs block P450 aromatase, decreasing tissue estrogen production. Two types of AIs have been approved for breast cancer: irreversible inhibitors and nonsteroidal inhibitors. Exemestane is an irreversible inhibitor that binds permanently to enzyme, extinguishing its regular catalytic function. Anastrozole is a nonsteroidal inhibitor that binds competitively but reversibly to inhibit aromatase activity. Many other AIs are also administered [73].

Hormone resistance is observed in certain BC patients due to the *novo* or natural resistance to estrogens or the acquisition of hormonal resistance that is attributed to progressive DNA mutations in tumor cells. In particular, the ER^- PR^- phenotype is with enhanced hormonal resistance. This phenomenon is more pronounced, despite ER expression, when cancer cells overexpress Her-2/neu, which is linked to poor prognosis. Many efforts have been directed toward inhibiting tumor hormonal resistance by simultaneously targeting estrogen and progesterone receptors with anti-Her2.

IMMUNE RESPONSE AND BREAST CANCER

Intervention in cancer by the immune system begins with the detection of transformed cells on proliferation—not with the attack and restraint of an established tumoral mass. In the late 1950s, Burnet proposed the *immunosurveillance* theory, in which immune system cells detect transformed cells, attacking them and eliciting an adaptive response to eliminate them or fail, leading to the formation of a tumoral mass and onset of cancer [74].

This process is more complex than originally believed, comprising not only a series of simple events but also alternative processes and stages, such as *immunoediting* and *immunosubversion*. The previous events can be explained by the paradox that greater immune cell infiltration in breast cancer neoplasias correlates with a worse disease prognosis and by the polarization that immune cells experience in the tumoral microenvironment, which induces the acquisition of a protumoral phenotype.

Despite a robust immune response in a tumor site, not all transformed cells are cleared, the remainder of which manage to avoid immune cell recognition and effector mechanisms. These cells proliferate, and in response, new immune mechanisms emerge (such as the humoral antitumor response), which imposes a new immunological selective pressure on transformed cells. This process is called *immunoediting* and leads to the generation of new clones of transformed cells with less immunogenity and an attenuated response to cytotoxic mechanisms of the immune system.

Such subsets have greater immune-modulatory properties, such as the ability to produce and secrete soluble factors that alter the tumoral microenvironment to become deleterious to the effector functions of immune cells and induce immunological tolerance and anergy to certain cell subsets. In this process, called immunosubversion, regulatory T lymphocytes intervene, in addition to several elements in the tumoral milieu, rendering immunosubversion the key strategy by which *successfully edited* transformed cells escape immune system pressure [75].

FUNCTION OF IMMUNE CELL SUBSETS IN CANCER REJECTION

Primary tumor growth is a complex process that involves many interactions between the tumor and surrounding tissue—a developing tumor influences and is in turn affected by its stroma, initiates angiogenesis, and interacts with the adaptive and innate immune systems. Primarily, in immune surveillance, innate immune cells intervene, such as NK and NKT cells, T$\gamma\delta$ lymphocytes, and antigen-presenting cells (APCs) (ie, macrophages and dendritic cells), as do cells of the adaptive response (cytotoxic and helper T cells and B lymphocytes).

Neutrophils

Neutrophils are the predominant leukocyte population in peripheral blood, constituting 50% to 70% of all leukocytes. Their function against bacterial infections is well established, and they also have antitumoral effects. Several studies have reported elevated neutrophil counts in the peripheral blood of patients with various tumors, such as breast cancer [76]. On activation, neutrophils attack transformed cells by producing reactive oxygen species (ROS), proteases, membrane-perforating agents, and other soluble cytotoxic and chemotactic factors, such as TNFα, IL-1β, IFN-γ, and defensins, which are highly toxic to many tumors. When a humoral response has been generated, neutrophils effect antibody-dependent cellular cytotoxicity (ADCC) through their receptors for FcRα and FcRγ [77].

NK cells

NK cells are effector lymphocytes of the innate immune system that control several types of tumors, viruses, and microbial pathogens by limiting their growth and dissemination, in part due to their cytolytic activity [78]. NK cells are one of the principal elements that conduct immunosurveillance, based on the association between low NK cell activity and increased cancer risk. NK cells perform constant surveillance of an organism's cells, sensing their surface molecules to determine whether they are healthy and *self*.

To this end, NK cells express many inhibitory and activating receptors. Some inhibitory receptors have affinity for major histocompatibility complex (MHC) class I molecules, which are highly polymorphic, even between individuals of the same species, and the expression of which can be altered in several pathologies. The chief activating receptors are NKG2D (natural killer group 2, member D) and the NCR (natural cytotoxicity receptors) family, including NKp30, NKp44, and NKp46; the ligands of these receptors are barely detectable in normal cells but are upregulated in response to cellular stress and transformation. Activating and inhibitory signals are integrated; thus, NK cells can recognize and discriminate between *self*, foreign, healthy, and unhealthy cells.

On activation, NK cells have 2 main effector mechanisms: 1. granule exocytosis, which involves perforin (a membrane-disrupting protein) and granzymes A and B (proteases that cleave and activate caspases); and 2. signaling from death receptor ligands, such as FasL (Fas ligand), TRAIL (TNF-related apoptosis-inducing ligand), and LT-α (lymphotoxin-alpha). In addition, activated NK cells produce many cytokines that control tumoral growth, such as IFNγ and TNF, which are antiproliferative, and shape the developing immune response. IFN-γ and TNF also promote dendritic cell maturation, and GM-CSF, G-CSF, and M-CSF induce proliferation and recruit myeloid cells.

NK cells and other antitumor immune cells distinguish normal cells from modified cancer cells through the expression of inhibitory and activating receptors that is triggered during target cell recognition. NK cells can kill target cells that lack or express low amounts of HLA class I molecules (expressed by normal cells) and

that express activating ligands—hallmarks of tumor cells. Notably, in tumors, the events that lead to transformation likely result in the downregulation of self-ligands and expression of stress-induced ligands by tumor cells, which can be recognized by NK cells [79, 80].

In certain animal models of cancer, mouse NK cells mediate the *in vivo* rejection of transplanted tumors, which depends on the expression of NK cell receptor ligands by the tumor [81]. In experimental models, NK cell-mediated elimination of tumor cells induces the development of tumor-specific T cell responses to the parental tumor, bridging the innate and adaptive immune responses [82, 83]. NK cells are also mediators of the antitumor effects of several recombinant cytokines (IL-2, IL-12, IL-18, and IL-21) [81]. An epidemiological study has demonstrated that low NK cell activity in peripheral blood is associated with increased cancer risk in adults [84]. $CD8^+$ cytotoxic T cells (CTLs) and NK cells are most likely effectors of successful antitumor immunity [85]. Although there is no evidence of a link between NK cell infiltrates and clinical outcomes in patients with breast cancer, the expression of NK cell ligands has a critical function in tumor immunoediting and the concomitant immune evasion in breast cancer [86].

The two principal subtypes of breast cancer are luminal and basal, based on specific patterns of ligand expression. Luminal breast cancers express lower levels of inhibitory and activating molecules compared with healthy breast tissue, suggesting poor stimulation of NK cell-mediated immunity and other components of the antitumor response. Basal breast cancers express high levels of inhibitory and activating ligands. The chief difference between basal and luminal cancers concerns disease evolution and clinical outcome. Only the luminal subtype expresses hormone receptors and is subdivided into luminal-A and luminal-B breast cancer, the latter of which is highly proliferative, resists hormone therapy, and has a poor prognosis.

The chief predictor of the evolution of luminal breast cancer is proliferation, which results from intrinsic genomic abnormalities and the proinflammatory environment.

Basal tumors have a poor prognosis compared with luminal tumors, and basal breast cancer is highly proliferative; thus, proliferation is not a predictor of its evolution. Certain subgroups of basal breast cancer have a relatively better prognosis, which might be attributed to the expression of genes that mediate antitumor immunity. Thus, the main predictor of outcome for basal subtypes is the antitumor immune response. The antitumor immune response differs between the 2 breast cancer subtypes.

Breast tumors have evolved to be undetected by antitumor immunity, at least at diagnosis; those with greater visibility have a better prognosis. Breast cancer cells have developed strategies to avoid recognition; phenotypic and functional analyses of NK cells from various stages of breast cancer have demonstrated that they function poorly in patients with invasive breast cancer, whereas in patients who are in long-term remission, these phenotypic changes disappear. It is possible that tumors become resistant to NK cell antitumor immunity or that their invasive characteristics and metastasis depend on the inhibition of NK cell-mediated antitumor activity [87].

Breast tumor cells use several mechanisms to resist NK cells, including inhibition through cell-cell contacts; secretion of inhibitory factors; and reorganization of the tumor microenvironment, notably with increased Treg recruitment and facilitation of mesenchymal stem cell growth [87]. The new surrounding environment supports tumor growth in situ or at the site of metastasis, which is protected from immune recognition; most of these modifications are effected through the massive secretion of TGFβ1, PGE2, and several metalloproteases that downregulate activating receptors and induce the shedding of homing molecules and surface beacons that are used as NK cell ligands [87].

In general, the imbalance in immune response favors immunosuppression in cancer patients, as evidenced by the decline in NK cell number in peripheral blood, a minor tumor infiltrate, and the decrease in toxicity. With regard to activating receptors, NKG2D, NKG2C, NKp30, NKp46, CD161, $CD56^{dim}$, CD16, DNAM-1, and CD69 have aberrant expression, whereas the inhibitory receptors CD158a, CD158b, and NKG2A are overexpressed [81, 88, 89].

NK T cells

Natural killer T (NKT) cells are similar to NK cells, except that they express a T cell receptor (TCR). NKT cells are classified into 4 groups. Types I and II are CD1d-restricted. Type I NKT cells, or invariant NKT cells (iNKT cells), develop in the thymus and are selected following rearrangement of the TCR α-chain gene and express a semi-invariant TCR (Vα14–Jα18 in mice; Vα24–Jα18 in humans). iNKT cells respond quickly to various glycolipids on CD1d, and strong iNKT cell agonists, such as α-galactosylceramide (αGalCer), induce the release of cytokines. On activation, NKT cells kill transformed cells by secreting

perforin and granzymes or through FasL and TRAIL. Notably, they can kill transformed cells that express CD1d on their surface directly, but such cells are a minority.

Because NKT cells express a TCR, they can be introduced to antigens and activated by APCs. iNKT cells respond directly to IL-12 in the B16 melanoma model and produce IFNγ, but they are dispensable for effective antitumor immunity. Once activated, iNKT cells upregulate CD40L, a costimulatory molecule, which promotes dendritic cell maturation. iNKT cells also produce IL-12 and IFN-γ, which together stimulate NK cells and cytotoxic T lymphocytes, increasing their cytotoxicity, enhance phagocytosis by macrophages and dendritic cells.

In humans, iNKT frequency declines in solid tumors, including melanoma and colon, lung, breast, and head and neck squamous cell carcinomas; increased iNKT cell numbers are associated with a better prognosis. Thus, these clinical findings are consistent with data from mouse studies and implicate iNKT cells in tumor immunosurveillance [90].

γδ lymphocytes

γδ T lymphocytes are a lymphoid subpopulation that is characterized by a TCR that comprises γ and δ chains instead of the typical αβ heterodimer of T lymphocytes. γδ T cells account for 2% to 5% of the peripheral T cell population, but they are common in organs and mucosa, constituting a firstline defense system at entry sites into an organism. γδ T cells are found primarily in the epithelia and mucosa-associated lymphoid tissue, particularly in respiratory, digestive, and reproductive organs, rendering them significant effectors against epithelial cancers and lymphoid malignancies.

The γδ T subpopulation identifies transformed cells by detecting ligands that are upregulated during stress through their TCRs and other receptors, such as NKG2D and Toll-like receptors (TLRs). These cells ingest, process, and present soluble antigens to other T lymphocytes. On activation, γδ T cells proliferate and exert their cytotoxic effects by releasing cytotoxic granules (perforin and granzymes) and through FasL and TRAIL; even without being activated, they can effect ADCC.

Activated γδ T cells recognize and lyse tumor cells *in vitro* and *in vivo*. NBP, PAg, IPP, interleukin-2 (IL-2), chemotherapeutic agents, and zoledronic acid (ZA) expand T cells and induce them to eliminate epithelial tumor cells *in vitro*. Further, these cells produce various cytokines and chemokines that have many effects on target cells, the surrounding healthy tissue, and other immune system cells.

For example, IFN-γ has antiproliferative effects and mediates T lymphocyte differentiation, directing a proinflammatory Th1 response. IL2 is an autocrine and paracrine stimulatory cytokine, and GM-CSF (granulocyte-macrophage colony stimulation factor) and CCL2 (or MCP-1, monocyte chemoattactant protein-1) stimulate the generation and recruitment of phagocytic cells. FGF-7 (fibroblast growth factor 7), KGF-1, and KFG-2 (keratinocyte growth factors 1 and 2) promote adjacent epithelial repair [91].

In murine models, repeated inoculation with activated γδ T cells delays or halts tumor growth in melanoma, prostate, bladder, and breast cancers [91]. Studies in mice have identified 3 pathways by which γδ T cells exert their anticancer activities: direct killing of transformed cells, early IFN-γ production, and a critical immunoregulatory mechanism [92].

Macrophages

Macrophages are phagocytic cells that reside in all tissues; during such events as tissue damage and infection, monocytes are recruited to the site of injury, where they differentiate into tissue macrophages. Macrophages express a variety of receptors on their surface, enabling them to recognize characteristic molecules in pathogens, stressed or necrotic cells, apoptotic bodies, and debris. Once they come into contact with these elements, macrophages ingest and process them in a vacuole called the phagosome. In addition, macrophages initiate and stimulate phagocytosis through their immunoglobulin receptors (IgRs), which bind to immunoglobulins (Igs) on cells and particles ina process called opsonization. Macrophages, as well as dendritic cells, are known as professional antigen-presenting cells (professional APCs) and constitute the link between innate and adaptive immunity.

After a pathogen or damaged cell is phagocytosed and processed, macrophages migrate toward the proximal draining lymph nodes, where they mature, enabling them to express costimulatory molecules (CD40, CD80, CD86) on their surface and activate T lymphocytes. Macrophages present peptide fractions of ingested bodies in MHC class II molecules to stimulate T helper cells. Eventually, through crosspresentation, macrophages also present peptides from phagocytosed bodies in the context of MHC class I molecules, through which cytotoxic T lymphocytes are activated.

Two classes of macrophages have been proposed, based on the state of activation: classically activated (M1) macrophages and alternatively activated (M2) macrophage. M1 macrophages arise on stimulation with IFN-γ alone or in concert with bacterial moieties, such as lipopolysaccharide (LPS) and cytokines [eg, tumor necrosis factor-α (TNF-α)]. In contrast, M2 macrophages are polarized by distinct stimuli and are further subdivided into M2a, M2b, and M2c macrophages. M2a macrophages are stimulated by the Th2 cytokines IL-4 and IL-13, and M2b macrophages are induced by immune complexes (ICs), LPS, TLRs, and IL-1 receptor antagonist (IL-1ra). M2c macrophages are induced by IL-10, transforming growth factor-β (TGF-β), and glucocorticoids (GCs). Macrophages have cytostatic and cytotoxic effects by producing reactive oxygen species (ROS), nitric oxide (NO), TNF-α, and type 1 IFN and expressing TRAIL and FasL. Moreover, they are a source of IL-12, which is necessary for the proliferation and activation of lymphocytes and other immune cells.

TAMs (tumor-associated macrophages) have an M2-like phenotype (characterized by the expression of CD163, IgG Fc fragment, C-type lectin domains, and heat shock proteins), but their function in cancer is unknown. In certain types of cancer, such as colorectal cancer, TAMs are proinflammatory and have antitumor activity, which improves the prognosis. M1 TAMs promote galcetin-3-expressing colon tumor cells, which induces greater TAM infiltration and amplifies the immune response against tumor cells. However, in most tumors, such as breast, prostate, ovarian, cervical, lung carcinoma, and cutaneous melanoma, TAMs are anti-inflammatory and correlate with a poor prognosis. Epidemiological studies have suggested that a macrophage-rich microenvironment promotes aggressive tumors with high metastatic potential.

Another significant aspect of tumor immunity is the tumor microenvironment, which harbors several chemoattractants, such as IL-4, IL-13, TGF-β, and IL-10, all of which effect the adoption of an M2 phenotype that can direct the differentiation of macrophages into the M2 phenotype and thus a poor prognosis [93].

Dendritic cells

Dendritic cells (DCs) are potent APCs that present antigens to antigen-specific naïve T cells and maintain the innate and adaptive immune responses. DCs are derived from bone marrow hematopoietic progenitor cells and circulate in the blood as immature precursors prior to migration into peripheral tissues. Like macrophages, it has been proposed that during infection or tissue damage, DCs are recruited by chemokines, such as CCL20 and CXCL12, where they identify pathogens, stressed and necrotic cells, apoptotic bodies, and debris through a wide range of surface receptors. The function APCs is similar to that of macrophages with regard to the uptake, processing, and presentation of antigens and costimulation in activating helper and cytotoxic T cells and naïve and memory B lymphocytes.

Further, on maturation, DCs produce cytokines that enhance and maintain the innate and adaptive immune responses. IL-12 increases the proliferation and activity of NK cells and T helper cells and, with IL-2, stimulates activated and memory B lymphocytes. IL-15 is required for the activation, proliferation, and survival of NK cells and cytotoxic T lymphocytes and types 1 and 2 IFN, which have stimulatory effects on NK cells, cytotoxic T lymphocytes, and other cells, induces the expression of TRAIL. Moreover, the IKDC (IFN-producing killer dendritic cell) subpopulation which phagocytoses and presents antigens and produces cytokines, kills tumor cells by expressing TRAIL and synthesizing perforin.

In tumor immunity, like macrophages, DCs are critical to antigen capture and presentation, but they are also vulnerable to polarizing signals by the tumor and tumor microenvironment. Suppression and repolarization of DC function in cancer patients is believed to contribute to the failure of antitumor immune responses and consequent disease progression. Subversion of tumor immunity by manipulating the tumor microenvironment and distribution and function of DC subsets is mediated by various tumor-derived and stromal factors, such as tumor cell-derived PGE2 and TGFβ.

Tumor-associated fibroblasts, through depletion of GMCSF from the tumor microenvironment, convert $CD11c^+$ dendritic cells into immunosuppressive macrophages. $CD11c^+DEC205^+MHC\text{-}II^+$ DCs are unable to present tumor antigens effectively and impede the robust priming of T cells by immunocompetent DCs. In addition, the tumor microenvironment induces cell death and accelerates apoptosis of DC precursors. The higher proportion of apoptotic blood DCs in patients with early-stage breast cancer versus healthy volunteers supports this mechanism of DC elimination in the tumor environment. The depletion of DCs in the advanced stages of tumor progression significantly delays tumor growth, allowing the immune system to regain control of tumors—also in the absence of any direct intervention against tumor cells [94, 95].

T helper lymphocytes

T helper lymphocytes reside in lymph nodes and other secondary lymphoid organs in a naïve state. On arrival to lymph nodes, mature APCs present a peptidic antigen that is associated with MHC class II molecules, the complex of which is recognized by the TCR (T cell receptor). Of the lymphocytes that interact with this APC, the cell whose TCR is specific for this complex receives the necessary costimulation (by surface molecules, such as CD80 and CD86) to proliferate, giving rise to clones that acquire an effector phenotype, depending on the microenvironment. Once activated, T helper cells migrate to where the antigen for which they are specific is expressed and produce cytokines and chemokines that modulate and orchestrate the immune response.

There are 3 types of T helper cells, depending on their cytokine profile. Th1 (T helper type 1) lymphocytes primarily secrete TNF-α and IFN-γ. Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Th17 cells secrete IL-17, IL-17F, IL-6, IL-22, and TNF-α. With regard to the antitumor response, the function of this cellular subpopulation is unknown, but Th1 lymphocytes appear to support such activity, releasing cytokines that contribute to the effector function of other immune cells having direct effects on tumor cells and the local microenvironment. The principal Th1 cytokine is IFN-γ, which is anti-proliferative and antiangiogenic, stimulates NK cells and cytotoxic T lymphocytes and increases their cytotoxicity, and activates macrophages and DCs. TNF-α also has antiproliferative effects and upregulates MHC I molecules in tumor cells and APCs, facilitating antigen presentation and cytotoxic susceptibility.

MHC II molecule expression is also higher in many cancer types, such as melanoma, lung and breast cancer, and osteosarcoma. This upregulation could also activate T helper cells and effect the simultaneous activation of other effector cells, such as cytotoxic lymphocytes. T helper cells induce apoptosis in tumor cells through one of several related mechanisms. Th cells can use the Fas/FasL pathway to induce apoptosis of tumor cells directly. For example, Th cells upregulate Fas expression on the lymphoma cell surface by binding CD40 on the tumor cell. Consequently, Th cells induce tumor cell apoptosis by FasL ligation. In other tumor types, Th cells use other apoptotic mechanisms, such as TNF-related apoptosis-inducing ligand (TRAIL). Th1 cells also employ a granzyme/perforin-dependent pathway to kill T lymphoma cells.

These cells can assume a regulatory phenotype that is similar to $CD4^+$ $CD25^+$ regulatory T cells, which can significantly impede the function of Th cells in the tumor microenvironment and prevent tumor eradication. Recent studies have reported the induction of Th-like regulatory T cells by coculture with natural regulatory T cells and the resulting production of immunosuppressive cytokines (TGF-β) that block the function of emerging antigen-specific Th cells [96, 97].

Cytotoxic lymphocytes (CTLs)

Cytotoxic T lymphocytes (CTLs) are activated by APCs in the lymph node, but unlike helper T cells, they require crosspresentation, because they recognize antigens that are associated with MHC class I molecules. MHC class I is on all nucleated cells and present self-antigens (or intracellular nonself) constitutively, which are central to recognizing healthy self, infected, transformed, and nonself cells. When cytotoxic T cells are activated, they undergo clonal expansion, acquire their effector phenotype, and migrate to where they are required. When CTLs encounter any cell that expresses the antigen that they target, they induce death by contact, through TRAIL, or through perforin and granzyme secretion. Further, CTLs produce TNF-α and IFN-γ, the effects of which have been described above.

In mouse and human, CTL function significantly in tumor rejection, inducing Fas receptor expression, the degradation of cellular FLICE/caspase-8 inhibitory protein (cFLIP) short protein, granzyme B-mediated lysis, and CD95-induced activation of the mitochondrial cell death pathway in tumor cells. An analysis of tumor-infiltrating T cells in murine tumor models and patient tumors have demonstrated that tumor-associated helper and cytotoxic T cells develop a functionally impaired effector/memory phenotype. CTLs infiltrate and eliminate their targets during early tumor growth but lose cytotoxic function on chronic exposure to the tumor microenvironment. CTLs retain the ability to secrete IL-2 and IFN-γ, but their lack of lytic properties is associated with a defect in cytoskeleton-mediated degranulation.

Tumor cells regulate the function of CTLs by impairing TCR signaling through various mechanisms, including signaling by the co-inhibitory molecules CTLA-4, PD-1, and BTLA; the accumulation and persistence of suppressor cells [Treg and myeloid-derived suppressor cells (MDSC)] and their byproducts. such as the immunosuppressive cytokines transforming growth factor (TGF)-β and

IL-10, and the catabolic enzymes iNOS, arginase, and ectonucleotidase; and tumor-produced immunosuppressive molecules, including PD-L1, TGF-β, IL-10, and indoleamine 2,3 dioxygenase (IDO). This suppression can be overridden by the presence of IL-12 and IFN-α, likely by inhibiting TGF-β signaling. However, it is unknown how IL-12 overcomes signaling by co-inhibitory molecules in the tumor microenvironment [98, 99].

Regulatory T cells (Tregs)

Regulatory T cells (Tregs) constitute 5% to 10% of $CD4^+$ T cells in the periphery and modulate the immune response with their suppressive effects [100]. Tregs are classified as natural regulatory T cells (nTregs) when they are generated in the thymus and induced regulatory T cells (iTregs) when they have differentiated from naive T cells in the periphery. Tregs are characterized by high expression of CD25 (IL-2Rα) and the transcription factor FoxP3, a significant regulator of Treg development and function [101]. The absence of FoxP3 in Scurfy mice effects an autoimmune and lymphoproliferative disease and causes immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans [102]. Helios is a transcription factor that was originally described as a marker of nTregs, but it has since been reported to be expressed in iTregs that have been generated *in vitro*.

Tregs express other markers, including CD5, $CD45RB^{lo}$, CTLA-4, PD-1, LAG-3, and GITR [100], all of which participate in their suppressive effects. Tregs inhibit APC activity through a contact-dependent mechanism that involves FasL/Fas- and PD1/B7-H1 interactions and a contact-independent mechanism that is mediated by IL-10, TGF-β, IL-27, and IL-35. Several phenotypically and functionally distinct iTreg subsets from the CD4 and CD8 lineage have been described. The most extensively studied populations are $IL\text{-}10^+$ T regulatory 1 (Tr1), TGF-β T helper (Th) 3, $CD4^+CD25^+$ nTreg-like, $CD8^+CD25^+$, and $CD8^+CD28^-$cells.

Tregs are generally hypothesized to promote cancer development by suppressing antitumor immune responses. Tregs can be activated by tumor-derived self-antigens or tumor-associated antigens and cause cancer by inhibiting the antitumor response. Elimination of Tregs by chemical or antibody depletion restores the immune response in several models, allowing the eradication of tumors [103]. In patients with pancreatic and breast cancer, increased levels of cells with a similar phenotype (positive for IL-10, TGF-β, and CTLA-4) have been detected in the PB, LNs, and tumor tissue.

$CD4^+CD25^+$Tregs suppress NK cell-mediated cytotoxicity in patients with various epithelial tumors, including lung, breast, and colorectal cancer. One of the proposed mechanisms of this phenomenon is the presence of the chemokine CCL22. Secreted by ovarian cancer cells and TAMs, CCL22 binds CCR4 on Tregs and forms a concentration gradient, inducing the egress of Tregs from draining LNs toward the CCL22-rich tumor microenvironment. CCL22 facilitates the encounter between DCs and activated antigen-specific T cells, suggesting that tumors have replicated this process to suppress activated effector cells. Tumor cells also induce Tregs directly through several factors, including CD70, cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), IL-10, galectin-1, and TGF-β.

$CD8^+$ regulatory cells and their $CD4^+$ counterparts were described in the early 1970s. Tregs from the $CD8^+$ lineage can develop intrathymically and in peripheral tissue. $CD8^+CD25^+FOXP3^+$CTLA nTregs have been identified in several studies in rodents and humans, and their activity is primarily cell-to-cell contact-dependent.

Recent reports have described $CD8^+$ Tregs in cancer patients. In prostate cancer patients, the $CD8^+$ Tregs are $CD25^+CD122^+FOXP3^+$ and $GITR^{+lo}$. Their suppressive effects are mediated by cell-to-cell contact and through unidentified soluble factors other than IL-10 or TGF-β. $CD8^+CD25^+FOXP3^+$ Tregs in colorectal cancer are positive for TGF-β. Tumor plasmacytoid DCs (pDCs) from ovarian cancer patients induce $CD8^+$ iTregs *in vitro*, which corroborates *ex vivo* data of the accumulation of $CD8^+$ Tregs in the ascites, draining LNs, and PB of these subjects. CD8+$CD28^-$ iTregs suppress through contact-dependent mechanisms, IL-10 secretion, and upregulation of the inhibitory immunoglobulin-like transcript (ILT) receptors ILT3 and ILT4 on APCs. The characterization and understanding of $CD8^+$ Tregs are in their infancy; thus, their subclassification and function are unsettled and will likely be modified and expounded on [104].

In humans and mouse models, a decrease in cytotoxic $CD8^+$ lymphocyte:Treg cell ratio in a tumor and draining lymph nodes is associated with a poor prognosis in several cancers, such as lung, liver, pancreas, breast, and ovary. However, this function can also limit inflammation and thus restrict tumor metastasis and development [105].

B lymphocytes

The function of B cells in cancer immunology is unknown, because there is evidence of their protumor and antitumor effects. The chief mechanism by which B cells exert their antitumoral effects that has been proposed is the production of antibodies against tumor antigens, which can facilitate the destruction of tumor cells by inducing ADCC, complement-mediated lysis, and phagocytosis. However, the efficacy of these antibodies has been debated, because they are typically detected at low concentrations and because many of the antigens are intracellular components.

Further, B lymphocytes modulate the immune response by acting as APCs and producing cytokines. As APCs, B cells express costimulatory molecules, such as CD80, CD86, and ICOS, activating helper and cytotoxic T lymphocytes. In addition, B lymphocytes can acquire an effector phenotype on activation by Th1 lymphocytes in the presence of IL-12, whereby B cells transform into effector B lymphocytes 1 (Be-1) that produce IL-12, IFN-γ, and TNF-α, further driving the Th1 response. Conversely, if B cells are activated by Th2 lymphocytes in the presence of IL-4, they acquire a Be-2 phenotype, which is associated with the production of IL-2, IL-4, and IL-13—all of which promote a Th2 immunity.

In solid tumors, such as melanomas, $CD27^+$ and $CD19^+$B cells are depleted, which is accompanied by B2-induced polarization of macrophages and TAMs through IL-10 production. In these B cells, the ability to upregulate IFN-γ and IL-2 in T cells and inhibit $CD4^+$ T cell-mediated help for CTLs is impaired.

There is new population of regulatory B cells (Bregs) that constitutes 1% to 2% of all $B220^+$ cells, subdivided into IL-10-producing $CD1d^{hi}CD5^+$B cells (B10 regulatory cells), B1b ($CD5^-CD1d^{hi}B220^{l\circ}CD11b^+IgM^+$) regulatory cells, and $CD19^+CD24^{hi}CD38^{hi}$B cells, that have been related to autoimmune diseases in mouse and humans. Bregs appear to use IL-10 as their chief effector molecule and are active in breast cancer lung metastasis. We have identified a unique and poorly proliferating B cell subset that resembles immature B2 cells phenotypically but expresses constitutively active Stat3 and high levels of IL2Rα (CD25), B7-H1, and CD81. These cells, designated tumor-evoked Bregs (tBregs), are required for lung metastasis, because breast cancer cells can not metastasize in their absence [106] (Fig. 2).

Mast cells

Mast cells (MCs) are tissue-resident immune sentinel cells that are found in most vascularized tissues in close proximity to blood vessels, nerves, smooth muscle, and epithelial cells. They are particularly abundant in sites that are exposed directly to the environment, such as the skin, airways, and the genitourinary and gastrointestinal tracts. MCs secrete a wide range of mediators with various activities that induce proinflammatory, anti-inflammatory, and immunosuppressive responses [107].

MCs are common in the tumor microenvironment in many human cancers, including breast cancer, and have been associated with tumor rejection and tumor promotion in experimental and clinical studies. Tumor-infiltrating MCs are derived from sentinel MC progenitor cells and those that are recruited to the tumor by tumor-derived chemoattractants, in response to which they release cytotoxic compounds or aid tumors indirectly by organizing its microenvironment through the secretion of mediators that favor angiogenesis or modulate immune responses [108].

Although the accumulation of MCs is linked to enhanced growth and invasion in certain human cancers [109], high MC infiltration has most often been associated with a good prognosis in breast cancer [110–115]. The presence of stromal mast cells correlated with a favorable prognosis in 187 breast carcinomas, and a high mast cell count was associated with small tumors, tubular differentiation, and hormone receptor expression [110]. There was also a negative correlation between the number of mast cells in regional lymph nodes and the presence of breast cancer metastases, suggesting an inhibitory function of mast cells against tumor growth [114].

A separate study confirmed the favorable prognostic significance of stromal mast cells in a cohort of 348 invasive breast carcinoma patients, demonstrating that the presence of stromal mast cells correlated negatively with tumor size but had no relationship with ER, PR, or HER2 status or tumor grade [112]. Further, high stromal mast cell counts in invasive breast cancer tissue are linked to low-grade tumors and ER positivity, factors that are associated with a favorable prognosis in breast cancer [111]. These results are supported by another study that reported that highly hormone-receptive tumors contain many MCs in the peritumoral area in invasive ductal breast cancer versus control cases and minimally hormone-receptive tumors.

Proliferation and ER, PR, and HER2 values indicate that a higher MC count is associated with a favorable

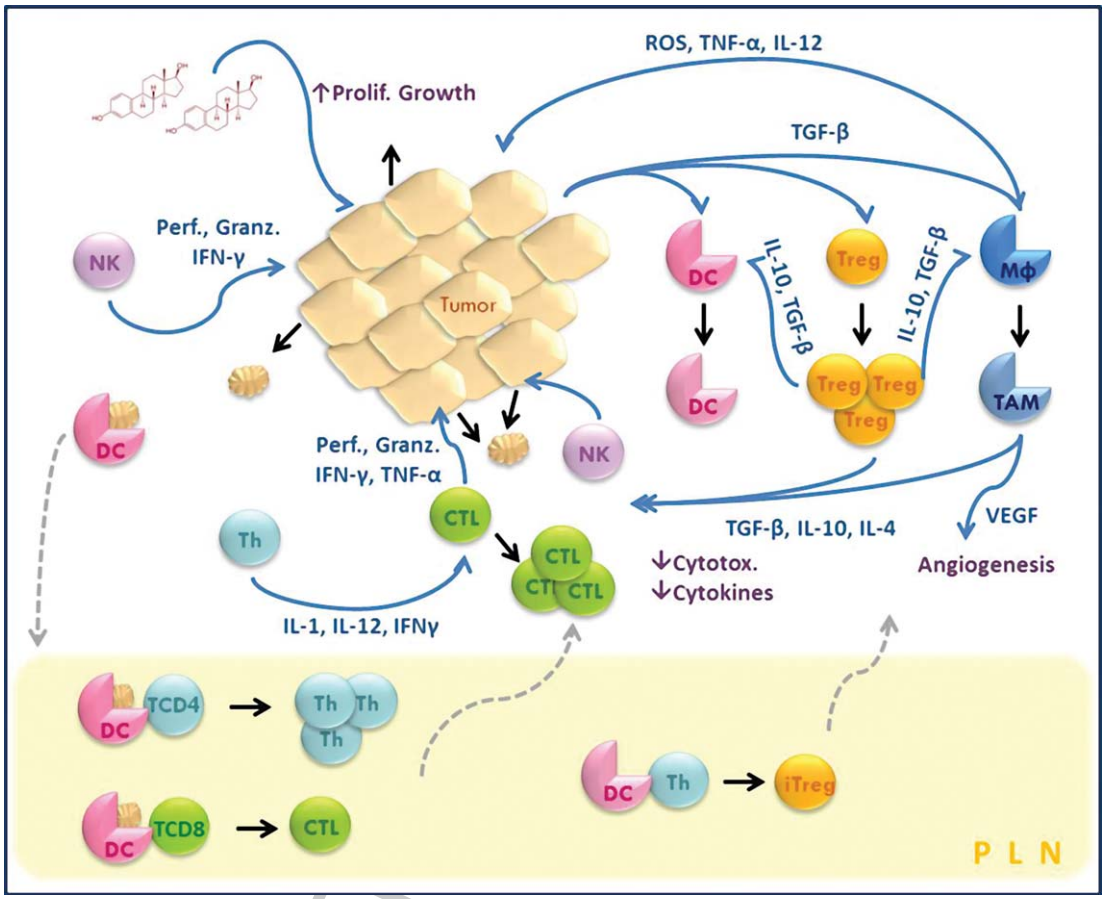

Fig. 2. Immunosurveillance and immunosubversion. Innate immunity elements are the first components to intervene. NK cells detect abnormal or stressed cells and attack them by secreting perforin and granzymes or by engaging death receptors, effecting tumor cell apoptosis. Apoptotic bodies are phagocytosed and processed by APCs (DCs or macrophages), which migrate to peripheral lymph nodes (PLNs) and present antigens to lymphocytes. Next, T helper and T cytotoxic lymphocyte clones arise and mediate the antitumoral response. Conversely, tumor cells modify the microenvironment by producing immunoregulatory factors, such as TGF-β, which alters cytotoxic cells and APC activity (leading to iTreg generation), promotes Treg proliferation, and converts macrophages into TAMs, which benefits a protumoral microenvironment.

prognosis. Moreover, MCs tend to accumulate around the cancerous area, which can be considered an attempt to impede the progression of anomalous tissue. MCs exhibit cytolytic activity against tumor cells [113]. In a larger study of 4444 breast tumors, stromal MCs predicted greater survival [115]. In contrast, a study noted that the number of MCs increased significantly in malignant compared with benign lesions in human breast biopsies [116]. A subsequent report found MCs counts to be negatively associated with ER/PR status, suggesting that higher MC counts are related to poor outcomes. However, there was no significant association between MC count and other parameters, including tumor size, lymph nodes status, and nuclear and histological grade [117].

Although mast cell phagocytosis has a significant function during a bacterial infection, its value in cancer has not been examined. Only one study has reported that MCs participate in the direct killing of breast tumor cells by phagocytosis and cytolysis [118].

MC-derived mediators have been implicated either in pro-tumorigenic or antitumorigenic functions. Histamine, the most extensively studied product of MCs, has been postulated to mediate tumor progression through histamine membrane receptors [119]. However, cimetidine, an antagonist of histamine receptor 2, had no effect on tumor cell proliferation in breast cancer patient, indicating that the presence of histamine and mast cells does not correlate with tumor cell proliferation in breast cancer [120].

MCs also participate in tumor angiogenesis, based on the release of several proangiogenic factors, of which tryptase, an MC-specific protease, is one of the most active. Tryptase-positive MCs have been linked

to angiogenesis in lymph nodes with micrometastases from breast cancer patients [121]. In addition, the number of tryptase-positive MCs correlates with angiogenesis in early breast cancer patients [122]. *In vitro* experiments have demonstrated that MC tryptase promotes the invasion and migration of breast cancer cells [123].

Conversely, MCs are a plentiful source of several proinflammatory cytokines and can have antitumorigenic effects, promoting the inhibition of tumor cell growth, tumor cell apoptosis, and inflammation by releasing proinflammatory cytokines, such as IL-1, IL-4, IL-6, and TNF-α—cytokines that inhibit the growth of breast cancer cells [124, 125]. In summary, the precise function of MCs in breast cancer is complex and remains undefined.

SEX STEROID REGULATION OF THE IMMUNE RESPONSE

The mammalian immune system distinguishes between self and nonself, using complex and evolving dynamic mechanisms that are essential for protection against pathogens. Initially, the immune system was believed largely to be self-regulated; however, it became clear that it coordinates bidirectionally with the neuroendocrine system [2]. The immune response, a homeostatic response that is under physiological control, maintains the integrity of cells in the body and tissue. Thus, efficient immunoendocrine communication implies the existence of afferent and efferent pathways that constitute a complex feedback system [126]. Sex-based differences in infection and immunity suggest that sex steroids underlie these disparities. Thus, in addition to the immune factors that regulate the complex immunoendocrine network, gender might have a significant function in shaping the immune response [127].

A reciprocal relationship between sex steroids and the immune system has been hypothesized for several years, and there is evidence that sex hormones influence the distribution and function of innate and adaptive immune cells. In addition, gender and sex steroids govern the development and prevalence of many human diseases. Thus, understanding the basis of differences in the immune response between genders is paramount for developing new approaches to prevent, diagnose, and treat infectious and autoimmune diseases.

In many sexually dimorphic species, sexual genotype is determined on conception, after which the organism's physiological and endocrinological development takes place, affecting many complex differences between males and females. Beginning in infancy and throughout reproductive age, these differences are based on the production, secretion, and circulating concentrations of estrogens, progesterone, and testosterone and are based primarily on the function and development of the hypothalamic-pituitary-gonadal (HPG) axis [1, 4]. The complex interaction between hormones that are produced by the HPG axis and other hormones, in addition to sex-independent gene products, determine the male and female phenotypes. Although individuals exhibit considerable variations, cyclic peaks of estrogen and progesterone and low androgen levels define the female phenotype. In contrast, low estrogen and progesterone and high androgen levels characterize the male phenotype [2].

Thus, we infer that in addition to their effects on sexual differentiation and reproduction, sex hormones govern the differences between sexes regarding their immune responses to the same antigenic stimulus. Although the first report on the presence of sex steroid receptors in the thymus was published in 1979 by Grossman et al., it was as early as 1898 when Italian scientist Cazolari observed that the thymii of rabbits that were castrated before sexual maturity were larger than in control animals. However, this finding went largely unnoticed. In 1940, Chiodi made a similar observation regarding the effects of castration on thymic weight. In addition, the finding that androgen replacement reverts castration-induced thymic hypertrophy strongly suggests that sex steroids mediated this effect.

Subsequently, immunological dimorphism was demonstrated, based on the finding that females of various species produce higher levels of circulating immunoglobulins and typically develop a stronger humoral response against infections. Females also produce a range of autoreactive antibodies more frequently. Increasing evidence of sexual dimorphism in the immune response in humans; compared with men, women mount more robust antibody and cell-mediated immune responses following an infection or vaccination. The incidence of most autoimmune diseases is also higher in women, but during pregnancy, many such diseases go into remission, only to flare up in the early postpartum period. Sexual hormones modulate variety wide range of phenomena with regard to the immune response, including thymocyte maturation and selection, cellular transit, lymphocyte proliferation, expression of MHC class II molecules and receptors, and cytokine production [4, 8, 128].

There are many effects of sex steroids on specific phenomena. For example, based on clinical and epidemiological findings, autoimmune diseases are more common in women than men and more frequent in women of reproductive age, suggesting that sex hormones govern these differences. According to data from the health systems of several countries, more than 75% of patients with autoimmune diseases are women, and in some extreme cases, specifically lupus erythematosus, 9 of 10 patients are women [129, 130]. Further, sex-associated disparities in the immune response are believed to cause the greater susceptibility of females to autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [131].

Sex-associated differences have also been observed in infectious diseases, including sexual dimorphism of the immune response and dimorphism with regard to to infection parameters [132]. Thus, sex-associated differences implicate sex steroids as modulators of the immune system.

As discussed, several immune and infectious diseases are believed to be influenced by gender and sex hormones. Recent evidence indicates that the control of Treg development is a key immune component of this sexual dimorphism

INTEGRATED REGULATION OF SEX STEROIDS, IMMUNE RESPONSE, AND BREAST CANCER

The sex hormones estrogen, testosterone, and progesterone affect the differentiation, maturation, and function of many immune cell types. Determination which cell types are targeted by sex steroids and increasing our understanding of the mechanisms by which these factors modify immune cell activity are critical to identify the basis of gender differences in autoimmune and infectious diseases and design the most appropriate therapeutic strategies.

Evidence suggests that the effects of sex steroids on the immune system cannot be generalized, because they can vary from immunosuppression to immunopotentiation, even with the same hormone. The variability in results might be explained by differences between systems, including whether a study is *in vitro* and *in vivo*, the dose of hormones (low vs high doses), age and sex hormone status, route and time of administration, hormone metabolism (eg, metabolism of testosterone to estrogen), and the effects of systemic treatment on the levels of other steroid hormones.

Experimental and clinical evidence in many models has linked sex steroid hormones, including estradiol, testosterone, and progesterone, and autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), SLE, and experimental autoimmune encephalomyelitis (EAE). Based on these observations, clinical trials that are based on hormone therapy and strategies to modulate autoimmunity through selective estrogen receptor modulators have been performed. However, treatment with sex steroids is a complicated clinical issue for which the risks and benefits must be weight. Additional basic information is essential to increase our understanding of the immune and neural implications of clinical used of sex steroids and sex steroid receptor-modulating drugs. Such studies will provide the basis for new therapeutic strategies for breast cancer.

TARGETED THERAPY

Targeted cancer therapy is directed against specific properties of cancer cells, including proteins that enable them to grow in rapidly or abnormally. Targeted therapies generally do not harm healthy cells. Some targeted therapies are antibodies, or targeted immune therapies.

Approximately 20% to 30% of breast cancers express HER2 (human epidermal growth factor receptor 2). In normal breast cells, HER2 receives signals that stimulate growth. But with an excess of HER2, breast cancer cells grow and divide too quickly. There are 2 antibodies against HER2 that allows cancer cells to receive chemical signals that signal them to grow, Herceptin (trastuzumab) and Perjeta (pertuzumab). Like Herceptin, Perjeta binds to HER2 on the surface of breast cancer cells and blocks growth signals. Herceptin reduces or stops the growth of breast cancer, while Perjeta, which is directed to a different region of HER2, complement Herceptin with regard to function.

Herceptin can damage the heart and its ability to pump blood, a risk that ranges between 5% and 30%. The risk of heart damage is greater when Herceptin is administered with other chemotherapy drugs that cause heart damage, such as adriamycin. In rare cases, Herceptin causes a severe allergic reaction, the symptoms of which include hives, and breathing difficulties due to sudden swelling and narrowing of the airways. Pulmonary toxicity—inflammation of lung tissue, low blood pressure, and possibly the accumulation of fluid around the lungs—is also rare.

In most cases, these reactions occur during the infusion or within the first 24 hours that the first dose

of Herceptin is given, such reactions develop less frequently within 1 week after the first dose. Occasionally, these adverse effects occur with or after the second dose. The reaction can be more severe when lung disease is concomitant with preexisting asthma or emphysema or if breast cancer has spread considerably to the lungs [133].

Mastectomy has become more refined and less intrusive, because in most cases, the muscles under the breasts are not removed. Lymph node removal and axillary lymph node dissection are treatment options if a biopsy demonstrates that the breast cancer has spread outside of the milk duct.

Prophylactic removal of the ovaries is a preventive surgery that decreases the amount of estrogen in the body, making it more difficult to stimulate the development of breast cancer [35].

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REFERENCES

[1] Arteaga M, Chavarria A, Morales Montor J. [Immunoneuroendocrine communication network and homeostasis regulation: The use of hormones and neurohormones as immunotherapy]. Rev Invest Clin. 2002; 54(6): 542-549. La red de comunicacion neuroinmunoendocrina y la regulacion de la homeostasis: el uso de hormonas y neurohormonas como inmunoterapia.

[2] Besedovsky HO, del Rey A. Immune-neuro-endocrine interactions: Facts and hypotheses. Endocr Rev. 1996; 17(1): 64-102.

[3] Besedovsky HO, del Rey A. The cytokine-HPA axis feedback circuit. Z Rheumatol. 2000; 59(Suppl 2II): 26-30.

[4] Grossman CJ, Roselle GA. The interrelationship of the HPG-thymic axis and immune system regulation. J Steroid Biochem. 1983; 19(1B): 461-467.

[5] Bottasso O, Morales-Montor J. Neuroimmunomodulation during infectious diseases: Mechanisms, causes and consequences for the host. Neuroimmunomodulation. 2009; 16(2): 65-67.

[6] Buckingham JC, Loxley HD, Christian HC, Philip JG. Activation of the HPA axis by immune insults: Roles and interactions of cytokines, eicosanoids, glucocorticoids. Pharmacol Biochem Behav. 1996; 54(1): 285-298.

[7] Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med. 1995; 332(20): 1351-1362.

[8] Da Silva JA. Sex hormones and glucocorticoids: Interactions with the immune system. Ann N Y Acad Sci. 1999; 876: 102-117; discussion 117-118.

[9] del Rey A, Besedovsky HO. The cytokine-HPA axis circuit contributes to prevent or moderate autoimmune processes. Z Rheumatol. 2000; 59(Suppl 2II): 31-35. Epub 2001/01/13.

[10] Derijk R, Berkenbosch F. The immune-hypothalamo-pituitary-adrenal axis and autoimmunity. Int J Neurosci. 1991; 59(1-3): 91-100.

[11] Liehr JG. Is estradiol a genotoxic mutagenic carcinogen? Endocr Rev. 2000; 21(1): 40-54.

[12] Bosland MC, Ford H, Horton L. Induction at high incidence of ductal prostate adenocarcinomas in NBL/Cr and Sprague-Dawley Hsd: SD rats treated with a combination of testosterone and estradiol-17 beta or diethylstilbestrol. Carcinogenesis. 1995; 16(6): 1311-1317.

[13] Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev. 1993; 15(1): 17-35.

[14] Russo J, Moral R, Balogh GA, Mailo D, Russo IH. The protective role of pregnancy in breast cancer. Breast cancer research: BCR. 2005; 7(3): 131-142.

[15] Cleary MP, Grossmann ME. Minireview: Obesity and breast cancer: The estrogen connection. Endocrinology. 2009; 150(6): 2537-2542.

[16] Rudel RA, Fenton SE, Ackerman JM, Euling SY, Makris SL. Environmental exposures and mammary gland development: State of the science, public health implications, and research recommendations. Environ Health Perspect. 2011; 119(8): 1053-1061.

[17] Silva E, Rajapakse N, Kortenkamp A. Something from "nothing"–eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Technol. 2002; 36(8): 1751-1756.

[18] Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology. 1993; 132(6): 2279-2286.

[19] Soto AM, Vandenberg LN, Maffini MV, Sonnenschein C. Does breast cancer start in the womb? Basic Clin Pharmacol Toxicol. 2008; 102(2): 125-133.

[20] Vihko RK, Apter DL. The epidemiology and endocrinology of the menarche in relation to breast cancer. Cancer Surv. 1986; 5(3): 561-571.

[21] Friedenson B. BRCA1 and BRCA2 pathways and the risk of cancers other than breast or ovarian. MedGenMed. 2005; 7(2): 60.

[22] Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature. 1997; 386(6627): 761-763.
[23] Rajan JV, Marquis ST, Gardner HP, Chodosh LA. Developmental expression of Brca2 colocalizes with Brca1 and is associated with proliferation and differentiation in multiple tissues. Dev Biol. 1997; 184(2): 385-401.
[24] Spillman MA, Bowcock AM. BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. Oncogene. 1996; 13(8): 1639-1645.
[25] Calderon-Garciduenas AL, Ruiz-Flores P, Cerda-Flores RM, Barrera-Saldana HA. Clinical follow up of mexican women with early onset of breast cancer and mutations in the BRCA1 and BRCA2 genes. Salud Publica de Mexico. 2005; 47(2): 110-115.
[26] Shen L, Yin ZH, Wan Y, Zhang Y, Li K, Zhou BS. Association between ATM polymorphisms and cancer risk: A meta-analysis. Mol Biol Rep. 2012; 39(5): 5719-5725.
[27] Lu X, Liu DP, Xu Y. The gain of function of p53 cancer mutant in promoting mammary tumorigenesis. Oncogene. 2013; 32(23): 2900-2906.
[28] Bogdanova N, Enssen-Dubrowinskaja N, Feshchenko S, Lazjuk GI, Rogov YI, Dammann O, Bremer M, Karstens JH, Sohn C, Dork T. Association of two mutations in the CHEK2 gene with breast cancer. Int J Cancer. 2005; 116(2): 263-266.
[29] Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. Mod Pathol. 2001; 14(7): 672-676.
[30] Caldeira JR, Prando EC, Quevedo FC, Neto FA, Rainho CA, Rogatto SR. CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. BMC Cancer. 2006; 6: 48.
[31] Bernstein L, Teal CR, Joslyn S, Wilson J. Ethnicity-related variation in breast cancer risk factors. Cancer. 2003; 97(1 Suppl): 222-229.
[32] Moss SF, Blaser MJ. Mechanisms of disease: Inflammation and the origins of cancer. Nat Clin Pract Oncol. 2005; 2(2): 90-97; quiz 1 p following 113.
[33] Pelekanou V, Leclercq G. Recent insights into the effect of natural and environmental estrogens on mammary development and carcinogenesis. Int J Dev Biol. 2011; 55(7-9): 869-878.
[34] Richert MM, Schwertfeger KL, Ryder JW, Anderson SM. An atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia. 2000; 5(2): 227-241.
[35] Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, Palacios J, Rakha EA, Richardson AL, Schmitt FC, Tan PH, Tse GM, Weigelt B, Ellis IO, Reis-Filho JS. Basal-like and triple-negative breast cancers: A critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol. 2011; 24(2): 157-167.
[36] Hu R, Dawood S, Holmes MD, Collins LC, Schnitt SJ, Cole K, Marotti JD, Hankinson SE, Colditz GA, Tamimi RM. Androgen receptor expression and breast cancer survival in postmenopausal women. Clin Cancer Res. 2011; 17(7): 1867-1874.
[37] Brisken C, Rajaram RD. Alveolar and lactogenic differentiation. J Mammary Gland Biol Neoplasia. 2006; 11(3-4): 239-248.
[38] Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 2006; 102(1-5): 89-96.
[39] Eden JA. Breast cancer, stem cells and sex hormones. Part 3: The impact of the menopause and hormone replacement. Maturitas. 2011; 68(2): 129-136.
[40] Sonnenschein C, Soto AM. An updated review of environmental estrogen and androgen mimics and antagonists. J Steroid Biochem Mol Biol. 1998; 65(1-6): 143-150.
[41] Kotsopoulos J, Narod SA. Androgens and breast cancer. Steroids. 2012; 77(1-2): 1-9.
[42] Liao DJ, Dickson RB. Roles of androgens in the development, growth, and carcinogenesis of the mammary gland. J Steroid Biochem Mol Biol. 2002; 80(2): 175-189.
[43] Zhou J, Ng S, Adesanya-Famuiya O, Anderson K, Bondy CA. Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression. FASEB J. 2000; 14(12): 1725-1730.
[44] Key T, Appleby P, Barnes I, Reeves G, Endogenous H, Breast Cancer Collaborative G. Endogenous sex hormones and breast cancer in postmenopausal women: Reanalysis of nine prospective studies. J Natl Cancer Inst. 2002; 94(8): 606-616.
[45] Missmer SA, Eliassen AH, Barbieri RL, Hankinson SE. Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. J Natl Cancer Inst. 2004; 96(24): 1856-1865.
[46] Zeleniuch-Jacquotte A, Toniolo P, Levitz M, Shore RE, Koenig KL, Banerjee S, Strax P, Pasternack BS. Endogenous estrogens and risk of breast cancer by estrogen receptor status: A prospective study in postmenopausal women. Cancer Epidemiol Biomarkers Prev. 1995; 4(8): 857-860.
[47] Baglietto L, Severi G, English DR, Krishnan K, Hopper JL, McLean C, Morris HA, Tilley WD, Giles GG. Circulating steroid hormone levels and risk of breast cancer for postmenopausal women. Cancer Epidemiol Biomarkers Prev. 2010; 19(2): 492-502.
[48] Gonzalez LO, Corte MD, Junquera S, Bongera M, Rodriguez JC, Vizoso FJ. Expression of androgen receptor and two androgen-induced proteins (apolipoprotein D and pepsinogen C) in ductal carcinoma in situ of the breast. Histopathology. 2007; 50(7): 866-874.
[49] Schippinger W, Regitnig P, Dandachi N, Wernecke KD, Bauernhofer T, Samonigg H, Moinfar F. Evaluation of the prognostic significance of androgen receptor expression in metastatic breast cancer. Virchows Arch. 2006; 449(1): 24-30.
[50] Shen R, Tao L, Xu Y, Chang S, Van Brocklyn J, Gao JX. Reversibility of aberrant global DNA and estrogen receptor-alpha gene methylation distinguishes colorectal precancer from cancer. Int J Clin Exp Pathol. 2009; 2(1): 21-33.
[51] Al-Azzawi F, Wahab M. Estrogen and colon cancer: Current issues. Climacteric. 2002; 5(1): 3-14.
[52] Hellberg D. Sex steroids and cervical cancer. Anticancer Res. 2012; 32(8): 3045-3054.
[53] Key TJ, Appleby PN, Reeves GK, Roddam AW, Helzlsouer KJ, Alberg AJ, Rollison DE, Dorgan JF, Brinton LA, Overvad K, Kaaks R, Trichopoulou A, Clavel-Chapelon F, Panico S, Duell EJ, Peeters PH, Rinaldi S, Fentiman IS, Dowsett M, Manjer J, Lenner P, Hallmans G, Baglietto L, English DR, Giles GG, Hopper JL, Severi G, Morris HA, Hankinson SE, Tworoger SS, Koenig K, Zeleniuch-Jacquotte A, Arslan AA, Toniolo P, Shore RE, Krogh V, Micheli A, Berrino F, Barrett-Connor E, Laughlin GA, Kabuto M, Akiba S, Stevens RG, Neriishi K, Land CE, Cauley JA, Lui LY, Cummings SR, Gunter MJ, Rohan TE, Strickler HD. Circulating sex hormones and breast cancer risk factors in postmenopausal

women: Reanalysis of 13 studies. Br J Cancer. 2011; 105(5): 709-722.
[54] Sieri S, Krogh V, Bolelli G, Abagnato CA, Grioni S, Pala V, Evangelista A, Allemani C, Micheli A, Tagliabue G, Schunemann HJ, Menard S, Berrino F, Muti P. Sex hormone levels, breast cancer risk, and cancer receptor status in postmenopausal women: The ORDET cohort. Cancer Epidemiol Biomarkers Prev. 2009; 18(1): 169-176.
[55] Bardou VJ, Arpino G, Elledge RM, Osborne CK, Clark GM. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. J Clin Oncol. 2003; 21(10): 1973-1979.
[56] Dunnwald LK, Rossing MA, Li CI. Hormone receptor status, tumor characteristics, and prognosis: A prospective cohort of breast cancer patients. Breast Cancer Res. 2007; 9(1): R6.
[57] Clemons M, Goss P. Estrogen and the risk of breast cancer. N Engl J Med. 2001; 344(4): 276-285.
[58] Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med. 2006; 354(3): 270-282.
[59] Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. Mol Endocrinol. 2005; 19(4): 833-842.
[60] Geisler J, Haynes B, Ekse D, Dowsett M, Lonning PE. Total body aromatization in postmenopausal breast cancer patients is strongly correlated to plasma leptin levels. J Steroid Biochem Mol Biol. 2007; 104(1-2): 27-34.
[61] Santen RJ, Santner SJ, Pauley RJ, Tait L, Kaseta J, Demers LM, Hamilton C, Yue W, Wang JP. Estrogen production via the aromatase enzyme in breast carcinoma: Which cell type is responsible? J Steroid Biochem Mol Biol. 1997; 61(3-6): 267-271.
[62] MacMahon B, Cole P, Lin TM, Lowe CR, Mirra AP, Ravnihar B, Salber EJ, Valaoras VG, Yuasa S. Age at first birth and breast cancer risk. Bull World Health Organ. 1970; 43(2): 209-221.
[63] Kobayashi S, Sugiura H, Ando Y, Shiraki N, Yanagi T, Yamashita H, Toyama T. Reproductive history and breast cancer risk. Breast Cancer. 2012; 19(4): 302-308.
[64] Dunbier AK, Anderson H, Ghazoui Z, Folkerd EJ, A'Hern R, Crowder RJ, Hoog J, Smith IE, Osin P, Nerurkar A, Parker JS, Perou CM, Ellis MJ, Dowsett M. Relationship between plasma estradiol levels and estrogen-responsive gene expression in estrogen receptor-positive breast cancer in postmenopausal women. J Clin Oncol. 2010; 28(7): 1161-1167.
[65] Secreto G, Zumoff B. Role of androgen excess in the development of estrogen receptor-positive and estrogen receptor-negative breast cancer. Anticancer Res. 2012; 32(8): 3223-3228.
[66] Devanesan P, Todorovic R, Zhao J, Gross ML, Rogan EG, Cavalieri EL. Catechol estrogen conjugates and DNA adducts in the kidney of male Syrian golden hamsters treated with 4-hydroxyestradiol: Potential biomarkers for estrogen-initiated cancer. Carcinogenesis. 2001; 22(3): 489-497.
[67] Chakravarti D, Mailander PC, Li KM, Higginbotham S, Zhang HL, Gross ML, Meza JL, Cavalieri EL, Rogan EG. Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene. Oncogene. 2001; 20(55): 7945-7953.
[68] Mitrunen K, Hirvonen A. Molecular epidemiology of sporadic breast cancer. The role of polymorphic genes involved in oestrogen biosynthesis and metabolism. Mutat Res. 2003; 544(1): 9-41.
[69] Ritchie MD, Hahn LW, Roodi N, Bailey LR, Dupont WD, Parl FF, Moore JH. Multifactor-dimensionality reduction reveals high-order interactions among estrogen-metabolism genes in sporadic breast cancer. Am J Hum Genet. 2001; 69(1): 138-147.
[70] Martinez-Ramirez OC, Perez-Morales R, Castro C, Flores-Diaz A, Soto-Cruz KE, Astorga-Ramos A, Gonsebatt ME, Casas L, Valdes-Flores M, Rubio J. Polymorphisms of catechol estrogens metabolism pathway genes and breast cancer risk in Mexican women. Breast. 2013; 22(3): 335-343.
[71] Hayashi SI, Eguchi H, Tanimoto K, Yoshida T, Omoto Y, Inoue A, Yoshida N, Yamaguchi Y. The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application. Endocr Cancer. 2003; 10(2): 193-202.
[72] Yoshida T, Eguchi H, Nakachi K, Tanimoto K, Higashi Y, Suemasu K, Iino Y, Morishita Y, Hayashi S. Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: Methylation of the gene and alteration of trans-acting factors. Carcinogenesis. 2000; 21(12): 2193-2201.
[73] Ray A, Ficek M. Immunomodulatory effects of antiestrogenic drugs. Acta Pharm. 2012; 62(2): 141-155.
[74] Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957; 1(5022): 779-786.
[75] Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: From immunosurveillance to tumor escape. Nat Immunol. 2002; 3(11): 991-998.
[76] Azab B, Bhatt V, Phookan J, Murukutla S, Kohn N, Terjanian T, Widmann W. Usefulness of the neutrophil-to-lymphocyte ratio in predicting short- and long-term mortality in breast cancer patients. Ann Surg Oncol. 2012; 19(1): 217-224.
[77] Dumitru C, Moses K, Trellakis S, Lang S, Brandau S. Neutrophils and granulocytic myeloid-derived suppressor cells: Immunophenotyping, cell biology and clinical relevance in human oncology. Cancer Immunol Immunother. 2012; 61(8): 1155-1167.
[78] Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008; 9(5): 503-510.
[79] Garrido F, Cabrera T, Aptsiauri N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: Implications for immunotherapy. Int J Cancer. 2010; 127(2): 249-256.
[80] Bottino C, Castriconi R, Moretta L, Moretta A. Cellular ligands of activating NK receptors. Trends Immunol. 2005; 26(4): 221-226.
[81] Levy EM, Roberti MP, Mordoh J. Natural killer cells in human cancer: From biological functions to clinical applications. J Biomed Biotechnol. 2011; 2011:676198.
[82] Kelly JM, Darcy PK, Markby JL, Godfrey DI, Takeda K, Yagita H, Smyth MJ. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. Nat Immunol. 2002; 3(1): 83-90.
[83] Sun JC, Lanier LL. Natural killer cells remember: An evolutionary bridge between innate and adaptive immunity? Eur J Immunol. 2009; 39(8): 2059-2064.
[84] Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: An 11-year follow-up study of a general population. Lancet. 2000; 356(9244): 1795-1799.
[85] Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: Immunoselection and immunosubversion. Nat Rev Immunol. 2006; 6(10): 715-727.

[86] Roberti MP, Mordoh J, Levy EM. Biological role of NK cells and immunotherapeutic approaches in breast cancer. Front Immunol. 2012; 3: 375.
[87] Mamessier E, Bertucci F, Sabatier R, Birnbaum D, Olive D. "Stealth" tumors: Breast cancer cells shun NK-cells anti-tumor immunity. Oncoimmunology. 2012; 1(3): 366-368.
[88] Konjevic G, Mirjacic Martinovic K, Jurisic V, Babovic N, Spuzic I. Biomarkers of suppressed natural killer (NK) cell function in metastatic melanoma: Decreased NKG2D and increased CD158a receptors on CD3-CD16+ NK cells. Biomarkers. 2009; 14(4): 258-270.
[89] Fuertes MB, Girart MV, Molinero LL, Domaica CI, Rossi LE, Barrio MM, Mordoh J, Rabinovich GA, Zwirner NW. Intracellular retention of the NKG2D ligand MHC class I chain-related gene A in human melanomas confers immune privilege and prevents NK cell-mediated cytotoxicity. J Immunol. 2008; 180(7): 4606-4614.
[90] Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nat Rev Immunol. 2012; 12(4): 239-252.
[91] Marquez-Medina D, Salla-Fortuny J, Salud-Salvia A. Role of gamma-delta T-cells in cancer. Another opening door to immunotherapy. Clin Transl Oncol. 2012; 14(12): 891-895.
[92] Girardi M. Immunosurveillance and immunoregulation by gammadelta T cells. J Invest Dermatol. 2006; 126(1): 25-31.
[93] Hao NB, Lu MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. Clin Dev Immunol. 2012; 2012:948098.
[94] Ma Y, Shurin GV, Gutkin DW, Shurin MR. Tumor associated regulatory dendritic cells. Semin Cancer Biol. 2012; 22(4): 298-306.
[95] Rutkowski MR, Stephen TL, Conejo-Garcia JR. Anti-tumor immunity: Myeloid leukocytes control the immune landscape. Cell Immunol. 2012; 278(1–2): 21-26.
[96] Knutson KL, Disis ML. Augmenting T helper cell immunity in cancer. Curr Drug Targets Immune Endocr Metabol Disord. 2005; 5(4): 365-371.
[97] Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. Immunol Rev. 2008; 222: 129-144.
[98] Egilmez N, Kilinc M. Tumor-resident CD8+ T-cell: The critical catalyst in IL-12-mediated reversal of tumor immune suppression. Arch Immunol Ther Exp. 2010; 58(6): 399-405.
[99] Weigelin B, Krause M, Friedl P. Cytotoxic T lymphocyte migration and effector function in the tumor microenvironment. Immunol Lett. 2011; 138(1): 19-21.
[100] Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring $CD4^+$ $CD25^+$ Foxp3+ regulatory T cells. Immunol Rev. 2006; 212: 60-73.
[101] Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat Immunol. 2003; 4(4): 330-336.
[102] Shevach EM. Regulatory T cells. Introduction. Seminars in Immunology. 2004; 16(2): 69-71.
[103] Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. Int J Cancer. 2010; 127(4): 759-767.
[104] Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res. 2010; 107: 57-117.
[105] Mougiakakos D, Johansson CC, Trocme E, All-Ericsson C, Economou MA, Larsson O, Seregard S, Kiessling R. Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. Cancer. 2010; 116(9): 2224-2233.
[106] Biragyn A, Longo DL. Neoplastic "Black Ops": Cancer's subversive tactics in overcoming host defenses. Semin Cancer Biol. 2012; 22(1): 50-59.
[107] Ribatti D, Crivellato E. The controversial role of mast cells in tumor growth. Int Rev Cell Mol Biol. 2009; 275: 89-131.
[108] Theoharides TC, Conti P. Mast cells: The Jekyll and Hyde of tumor growth. Trends Immunol. 2004; 25(5): 235-241.
[109] Ribatti D, Crivellato E. Mast cells, angiogenesis, and tumour growth. Biochim Biophys Acta. 2012; 1822(1): 2-8.
[110] Aaltomaa S, Lipponen P, Papinaho S, Kosma VM. Mast cells in breast cancer. Anticancer Res. 1993; 13(3): 785-788.
[111] Amini RM, Aaltonen K, Nevanlinna H, Carvalho R, Salonen L, Heikkila P, Blomqvist C. Mast cells and eosinophils in invasive breast carcinoma. BMC Cancer. 2007; 7: 165.
[112] Dabiri S, Huntsman D, Makretsov N, Cheang M, Gilks B, Bajdik C, Gelmon K, Chia S, Hayes M. The presence of stromal mast cells identifies a subset of invasive breast cancers with a favorable prognosis. Mod Pathol. 2004; 17(6): 690-695.
[113] della Rovere F, Granata A, Familiari D, D'Arrigo G, Mondello B, Basile G. Mast cells in invasive ductal breast cancer: Different behavior in high and minimum hormone-receptive cancers. Anticancer Res. 2007; 27(4B): 2465-2471.
[114] Naik R, Pai MR. Mast cell numbers in lymph node lesions. Indian J Pathol Microbiol. 1998; 41(2): 153-156.
[115] Rajput AB, Turbin DA, Cheang MC, Voduc DK, Leung S, Gelmon KA, Gilks CB, Huntsman DG. Stromal mast cells in invasive breast cancer are a marker of favourable prognosis: A study of 4,444 cases. Breast Cancer Res Treat. 2008; 107(2): 249-257.
[116] Kashiwase Y, Morioka J, Inamura H, Yoshizawa Y, Usui R, Kurosawa M. Quantitative analysis of mast cells in benign and malignant breast lesions. Immunohistochemical study on formalin-fixed, paraffin-embedded tissues. Int Arch Allergy Immunol. 2004; 134(3): 199-205.
[117] Kwon GY, Lee SD, Park ES. Mast cell and macrophage counts and microvessel density in invasive breast carcinoma-comparison analysis with clinicopathological parameters. Cancer Res Treat. 2005; 37(2): 103-108.
[118] Della Rovere F, Granata A, Monaco M, Basile G. Phagocytosis of cancer cells by mast cells in breast cancer. Anticancer Res. 2009; 29(8): 3157-3161.
[119] Reynolds JL, Akhter JA, Magarey CJ, Schwartz P, Adams WJ, Morris DL. Histamine in human breast cancer. Br J Surg. 1998; 85(4): 538-541.
[120] Bowrey PF, King J, Magarey C, Schwartz P, Marr P, Bolton E, Morris DL. Histamine, mast cells and tumour cell proliferation in breast cancer: Does preoperative cimetidine administration have an effect? Br J Cancer. 2000; 82(1): 167-170.
[121] Ribatti D, Finato N, Crivellato E, Guidolin D, Longo V, Mangieri D, Nico B, Vacca A, Beltrami CA. Angiogenesis and mast cells in human breast cancer sentinel lymph nodes with and without micrometastases. Histopathology. 2007; 51(6): 837-842.
[122] Ranieri G, Ammendola M, Patruno R, Celano G, Zito FA, Montemurro S, Rella A, Di Lecce V, Gadaleta CD, Battista De Sarro G, Ribatti D. Tryptase-positive mast cells correlate with angiogenesis in early breast cancer patients. Int J Oncol. 2009; 35(1): 115-120.
[123] Xiang M, Gu Y, Zhao F, Lu H, Chen S, Yin L. Mast cell tryptase promotes breast cancer migration and invasion. Oncol Rep. 2010; 23(3): 615-619.

[124] Gooch JL, Christy B, Yee D. STAT6 mediates interleukin-4 growth inhibition in human breast cancer cells. Neoplasia. 2002; 4(4): 324-331.
[125] Shen WH, Zhou JH, Broussard SR, Freund GG, Dantzer R, Kelley KW. Proinflammatory cytokines block growth of breast cancer cells by impairing signals from a growth factor receptor. Cancer Res. 2002; 62(16): 4746-4756.
[126] Armstrong MD, Klein JR. Immune-endocrine interactions of the hypothalamus-pituitary-thyroid axis: Integration, communication and homeostasis. Arch Immunol Ther Exp (Warsz). 2001; 49(3): 231-237.
[127] Chikanza IC, Grossman AB. Reciprocal interactions between the neuroendocrine and immune systems during inflammation. Rheum Dis Clin North Am. 2000; 26(4): 693-711.
[128] Bebo BF, Jr., Fyfe-Johnson A, Adlard K, Beam AG, Vandenbark AA, Offner H. Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. J Immunol. 2001; 166(3): 2080-2089.
[129] Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol. 1997; 84(3): 223-243.
[130] Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. Autoimmun Rev. 2003; 2(3): 119-125.
[131] Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update. 2005; 11(4): 411-423.
[132] Zuk M, McKean KA. Sex differences in parasite infections: Patterns and processes. Int J Parasitol. 1996; 26(10): 1009-1023.
[133] Fang L, Barekati Z, Zhang B, Liu Z, Zhong X. Targeted therapy in breast cancer: What's new? Swiss Med Wkly. 2011; 141: w13231.