UNIVERSIDAD COMPLUTENSE DE MADRID

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TESIS DOCTORAL

El receptor cannabinoide 2 en el microambiente inmunitario tumoral: papel en la progresión del cáncer de mama

Cannabinoid receptor 2 in the tumour immune microenvironment: role in breast cancer progression

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

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RESUMEN

El cáncer ya no es considerado una enfermedad en la que un grupo de células transformadas crece de manera descontrolada, si no un complejo ecosistema en el que las células tumorales interaccionan con su entorno y reciben señales que determinarán su destino, es decir, si sobreviven, proliferan y migran a otros órganos. Este ecosistema, que recibe el nombre de microambiente tumoral, es cada vez más reconocido como un jugador clave en la progresión del cáncer y también como una fuente de potenciales biomarcadores y dianas terapéuticas. Este microambiente consta de componentes celulares y no celulares, entre los que destacan las células inmunitarias por su papel pro- o antitumoral dependiente del contexto.

El cáncer de mama, la principal causa de muerte por cáncer en mujeres en el mundo, se considera poco inmunogénico por su relativamente bajo contenido de células inmunitarias infiltrantes y una activación limitada de vías inmunitarias. Sin embargo, cada vez hay más evidencias de que el sistema inmunitario juega un papel fundamental en la progresión de esta enfermedad, especialmente en los subtipos más agresivos como el cáncer de mama triple negativo. Las diferentes poblaciones inmunitarias expresan en mayor o menor medida el receptor de cannabinoides tipo 2 (CB_2R), uno de los receptores canónicos del sistema endocannabinoide, un complejo sistema de comunicación celular que participa en el mantenimiento de la homeostasis en numerosos procesos biológicos. Tanto la expresión como la activación de CB_2R en células inmunitarias han demostrado ejercer efectos inmunosupresores. Pero CB_2R también es la diana a través de la cual los cannabinoides, compuestos lipídicos derivados de la planta *Cannabis sativa*, desencadenan potentes respuestas antitumorales en modelos preclínicos de cáncer de mama. A pesar de este conocimiento, el papel de CB_2R en el microambiente tumoral de la mama y las consecuencias de su activación farmacológica en este contexto siguen siendo poco comprendidas. Además, la limitada bibliografía disponible aporta información contradictoria, evidenciando que este es un campo que requiere más investigación y convirtiéndolo en el objetivo principal de esta tesis.

En primer lugar, analizamos la expresión de CB_2R en células inmunitarias tanto *in silico* como *in vivo* y observamos que CB_2R es muy expresado por las células B y, sorprendentemente, por las células T citotóxicas. *In vivo* también hemos observado que los niveles de CB_2R en las células infiltrantes del tumor difieren significativamente de los observados en estado basal, destacando una expresión notablemente baja de CB_2R en las células T citotóxicas dentro del tumor. Además, encontramos que CB_2R regula la expresión de marcadores de inmunovigilancia, lo que sugiere un papel en la modulación de la respuesta inmunitaria antitumoral.

En segundo lugar, mediante el uso de modelos inmunocompetentes múridos basados en xenoinjertos, determinamos que la expresión de CB_2R en el estroma no tiene un impacto significativo en la iniciación y progresión de los tumores Luminal B o con sobreexpresión de HER2. Sin embargo, CB_2R sí tendría un papel pro-tumoral en cáncer de mama triple negativo, acelerando el crecimiento tumoral probablemente al generar un microambiente menos favorable para el desarrollo de respuestas antitumorales efectivas (es decir, con una menor infiltración de células asociadas con buen pronóstico en cáncer de mama y menor expresión de marcadores de inmunovigilancia). Además, en este subtipo tumoral, CB_2R

también tendría un papel pro-metastásico favoreciendo la generación de metástasis pulmonares sin alterar significativamente la infiltración inmunitaria en las lesiones metastásicas ya establecidas.

Por último, evaluamos el efecto de los cannabinoides sobre las células inmunitarias, observando respuestas de tipo inmunosupresor principalmente debido a la reducción de la viabilidad celular y a la interferencia con la vía de señalización JAK/STAT. Estos efectos son independientes del estado de activación de las células inmunitarias y, lo que es más sorprendente, no están mediados por CB_2R. A pesar de esta inmunomodulación negativa, en un contexto donde interactúan células inmunitarias y cancerosas (como ocurriría en un tumor), la acción antitumoral de los cannabinoides predominaría sobre el efecto inmunosupresor, lo que sugiere que estos compuestos podrían ser agentes antitumorales seguros y efectivos.

ABSTRACT

Cancer is no longer considered a disease in which a group of transformed cells grows uncontrollably, but rather a complex ecosystem where tumour cells interact with their surroundings and receive signals that determine their fate – whether they survive, proliferate, and spread to other organs. This ecosystem, known as the tumour microenvironment, is increasingly being recognised as a key player in tumour progression and a source of potential biomarkers and therapeutic targets. This microenvironment comprises cellular and non-cellular components, among which immune cells stand out for their context-dependent pro- or antitumoural roles.

Breast cancer, the leading cause of cancer-related female mortality worldwide, is considered poorly immunogenic due to its relatively low content of infiltrating immune cells and limited activation of immune pathways. However, there is increasing evidence demonstrating that the immune system plays a significant role in disease progression, especially in the most aggressive subtypes such as triple-negative breast cancer. Different immune cell populations express varying levels of cannabinoid receptor 2 (CB_2R), one of the canonical receptors of the endocannabinoid system, a complex cellular communication network involved in maintaining homeostasis in various physiological processes. Both expression and activation of CB_2R in immune cells have been associated with immunosuppressive effects. However, CB_2R is also the target through which cannabinoids, lipid compounds derived from the plant *Cannabis sativa*, elicit potent antitumoural responses in preclinical models of breast cancer. Despite this knowledge, the role of CB_2R within the breast tumour microenvironment and the consequences of its pharmacological activation in this context remain poorly understood. Furthermore, the limited available literature reports contradictory findings, highlighting that this is a field that requires further investigation, and establishing this as the main objective of the present thesis.

First, we analysed CB_2R expression in immune cells both *in silico* and *in vivo* and observed that CB_2R is highly expressed by B cells and, surprisingly, by cytotoxic T cells. *In vivo*, we also observed that CB_2R levels in tumour-infiltrating immune cells differ significantly from those observed basally, with a strikingly low expression of CB_2R in cytotoxic T cells within the tumour. Furthermore, we found that CB_2R regulates the expression of immunosurveillance markers within the tumour microenvironment, suggesting a role in the modulation of the antitumour immune response.

Second, by using murine xenograft-based immunocompetent models of breast cancer, we determined that CB_2R expression in the stroma does not significantly impact the initiation and progression of Luminal B or HER2-enriched tumours. However, in triple-negative breast cancer, CB_2R exerts a pro-tumoural role, accelerating tumour growth most probably by generating a microenvironment that is less prone to effective antitumour immune responses (i.e., with reduced infiltration of cells associated with improved prognosis and decreased expression of immunosurveillance markers). Moreover, in this tumour subtype, CB_2R would also have a pro-metastatic role, favouring the generation of lung metastasis without significantly altering immune infiltration in already established metastatic lesions.

Finally, we evaluated the effect of cannabinoids on immune cells, observing immunosuppressive-like responses mainly due to the reduction in cell viability and to interference with the JAK/STAT signalling pathway. These effects are independent of the immune cell activation state and, surprisingly, not driven by CB_2R. However, in a context where immune and cancer cells interact (as would occur in a tumour), the antitumour action of cannabinoids would predominate over the immunosuppressive effect, suggesting that these compounds may be safe and effective antitumour agents.

ABBREVIATIONS

2-AG: 2-arachidonoylglycerol

AA: arachidonic acid

ADCC: antibody-dependent cellular cytotoxicity

AEA: N-arachidonoylethanolamine / anandamide

APCs: antigen-presenting cells

BC: breast cancer

BSA: bovine serum albumin

(B)T(I)ME: (breast) tumour (immune) microenvironment

CAFs: cancer-associated fibroblasts

CB_1R: cannabinoid receptor 1

CB_2R: cannabinoid receptor 2

CBD: cannabidiol

CBRs: cannabinoid receptors

CCL/CCR: C-C motif chemokine ligand / C-C chemokine receptor

CINV: chemotherapy-induced nausea and vomiting

ConA: Concanavalin A

COX: cyclooxygenase

CTLs: cytotoxic T cells

CXCL/CXCR: C-X-C motif chemokine ligand / C-X-C chemokine receptor

DAGL: diacylglycerol lipase

DCs: dendritic cells

DFS: disease-free survival

DICE: Database of Immune Cell Expression project

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethylsulfoxide

DTCs: disseminated tumour cells

ECM: extracellular matrix

ECS: endocannabinoid system

(T)ECs: (tumour-associated) endothelial cells

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

EMT: epithelial-to-mesenchymal transition

ER: oestrogen receptor

FAAH: fatty acid amide hydrolase

FACS: Fluorescence-Activated Cell Sorting

FBS: fetal bovine serum

FDA: Food and Drug Administration

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

GPCR: G-protein coupled receptor

GrzB: Granzyme B

GTEx: Genotype-Tissue Expression

HER2: human epidermal growth factor receptor 2

HPA: Human Protein Atlas

ICIs: immune checkpoint inhibitors

ICs: immune cells

IFNGR: interferon-γ receptor

IFN-γ: interferon-γ

IL: interleukin

ImmGen: Immunological Genome Project

JAK: Janus Kinase

KO: knockout

LOX: lysyl oxidase

MAGL: monoacylglycerol lipase

MDSCs: myeloid-derived suppressor cells

MHC: major histocompatibility complex

MMP: matrix metalloproteinase

NAPE-PLD: N-arachidonoylphosphatidylethanolamine phospholipase D

NK: Natural killer cells

NKT: Natural killer T cells

NO: nitric oxide

NSCLC: non-small cell lung cancer

o/n: overnight

OS: overall survival

PBMC: peripheral blood mononuclear cell

PBS: phosphate saline buffer

pCR: pathological complete response

PCR: polymerase chain reaction

PD-1: programmed cell death protein 1

PD-L1: programmed death ligand 1

PFS: progression-free survival

PMN: pre-metastatic niche

PPAR: proliferator-activated receptor

PR: progesterone receptor

PTP1B: Protein Tyrosine Phosphatase 1B

PVDF: polyvinylidene fluoride

RBC: red blood cells

(sc)RNAseq: (single-cell) RNA sequencing

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute

RT: room temperature

SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis

STAT: Signal Transducer and Activator of Transcription

TAMs: tumour-associated macrophages

TBST: Tris-buffer saline Tween 20

TC-PTP: T-cell protein tyrosine phosphatase

TCR: T cell receptor

TGF-β: transforming growth factor β

Th1/Th2: Type 1/2 helper T cell

THC: Δ^9-tetrahydrocannabinol

TILs: tumour-infiltrating lymphocytes

TIMP-1: tissue inhibitor of matrix metalloproteinase-1

TLS: tertiary lymphoid structures

TMB: tumour mutation burden

TNBC: triple-negative breast cancer

TNF-α: tumour necrosis factor-α

Tregs: regulatory T cells

TRPV: transient receptor potential vanilloid

VEGF: vascular endothelial growth factor

WB: Western blot

WT: wild type

INTRODUCTION

BREAST CANCER

Breast cancer (BC) is a major global health problem as it is one of the most common malignancies diagnosed in women and the second leading cause of cancer-related female mortality worldwide (Giaquinto et al., 2024).

EPIDEMIOLOGY

Overall, BC mortality continues to fall, but incidence continues to rise, and it is predicted to increase to over 3 million new cases per year by 2040 due to growth and aging of the population (Arnold et al., 2022). However, there are significant geographical and demographic differences: while incidence is higher in countries with high human development index, probably due to greater capacity of early detection (for example, screening mammogram) and a higher prevalence of risk factors (late age at first pregnancy, less breastfeeding, hormone replacement therapy, alcohol consumption, physical inactivity, etc.), mortality is higher in low- and middle-income countries due to limited access to health care (H. Xu & Xu, 2023). Differences in tumour biology may also contribute to the ethnic disparity: for example, black women are twice as likely to be diagnosed with triple negative breast cancer (TNBC) and also appear to be less sensitive to neoadjuvant chemotherapy (Giaquinto et al., 2024).

Although most cases are sporadic, 5-10 % of BC is hereditary. Mutations in *BRCA1* and *BRCA2*, genes encoding proteins involved in DNA repair, account for 35 % and 25 % of hereditary cases, respectively. Other genes associated with hereditary BC risk are related to cell cycle control and maintenance of genomic integrity, such as *PTEN*, *TP53*, *STK11*, *ATM* and *PALB2* (Harbeck et al., 2019).

CLASSIFICATION

BC is a highly heterogeneous disease with variable histological and biological characteristics that determine its clinical behaviour and response to treatment. Thus, classification is needed to stratify patients and facilitate oncological decision making.

According to histological features, BC can be classified as *in situ* or invasive carcinoma, depending on whether it infiltrates or not adjacent tissue. Within invasive carcinomas, the most common histotype (75-80 %) is invasive carcinoma of no specific type (formerly invasive ductal carcinoma not otherwise specified), which is characterized by its marked heterogeneity, making it unsuitable for classification into any of the special histotypes. The latter group includes lobular carcinoma (the most common, accounting for 10-14 % of the total BC cases) and other rare subtypes such as tubular, mucinous, medullary, etc. (WHO, 2019).

In addition, histological grade classification (i.e., the degree of tumour differentiation) is widely used in clinical practice due to its strong prognostic value. The Nottingham Histologic Score System (NGS), also known as the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system, classifies BC into three grades according to the following parameters: glandular/tubular formation, degree of nuclear pleomorphism and mitotic index. According to this, Grade 1 includes well-differentiated (and therefore poorly aggressive) tumours [with high homology to the normal breast (**Box 1**)], while Grade 3

represents poorly differentiated (and therefore highly aggressive) tumours (Bloom & Richardson, 1957; Elston & Ellis, 1991).

Box 1. The mammary gland anatomy

The mammary gland is composed of a network of lobules and ducts formed by epithelial cells surrounded by a variety of stromal cells and connective tissue. This ductal-lobular network consists of two concentric layers; the one closest to the lumen is composed of luminal secretory epithelial cells, which undergo functional differentiation during pregnancy to produce milk, while the one closest to the basal membrane consist of basal myoepithelial cells with contractile capacity.

Non-transformed breast tissue also harbours immune cells, localised predominantly among the ductal epithelial cells rather than in the stroma and fat (**Figure B1**). Collectively, these cellular populations play a crucial role in morphogenesis (mammary gland development in shape and structure) and in post-lactational involution (regression to a pre-pregnant state). In addition, they contribute to defence by providing barrier immunity, particularly during lactation as it is the stage of highest predisposition to infection by bacteria (Dawson et al., 2020; Jaquish et al., 2024; Plaks et al., 2015; Rainard et al., 2022).

Characterisation of this immune microenvironment in the non-transformed mammary gland offers critical insights for BC prevention strategies, enhanced risk prediction models and mechanistic understanding of carcinogenesis control.

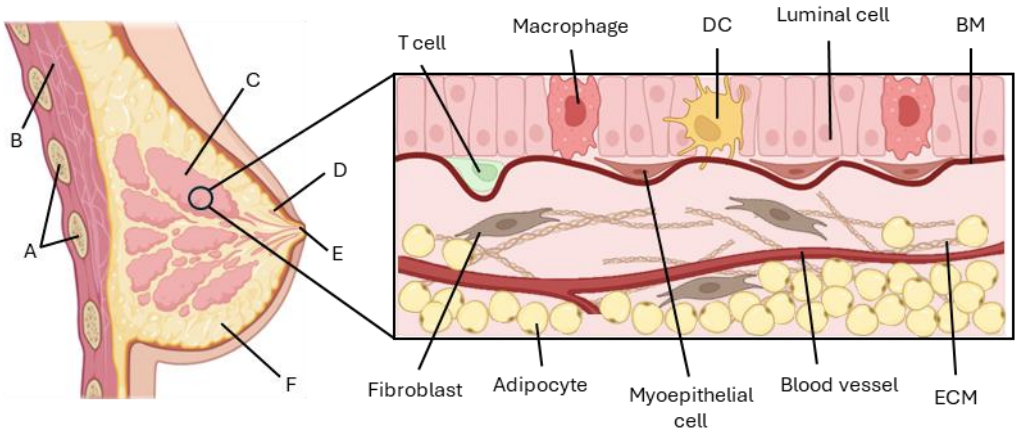

Figure B1. Mammary gland anatomy. On the left, sagittal cut of the breast. A: ribs, B: chest muscle; C: lobule; D: duct; E: nipple; F: fat. On the right, transversal cut of a non-transformed mammary duct showing the distribution of immune cells, primarily located within the ductal epithelium, and other stromal cells. BM: basement membrane; DC: dendritic cell; ECM: extracellular matrix.

Despite their prognostic value, these classifications do not fully reflect the heterogeneity of BC. In recent years, the use and development of high-throughput technologies (such as microarray-based technology) has allowed classification to be refined by detailed biological characterisation of genomic alterations. Thus, in the early 2000s, Perou, Sørlie and colleagues (Perou et al., 2000; Sørlie et al., 2001) defined 5 intrinsic subtypes by hierarchical clustering, with differences in incidence and clinical and pathological characteristics (**Figure I1**): luminal A, luminal B, HER2-enriched, basal-like and normal-like. Due to the controversy surrounding the latter, which is considered to be more of an artefact resulting from contamination with normal epithelial cells of a low malignant content tumour, it will not be further discussed. Subsequent studies have identified additional rare subtypes, including claudin low, molecular apocrine and interferon rich, but their identification as separated entities remains complex (Tsang & Tse, 2020).

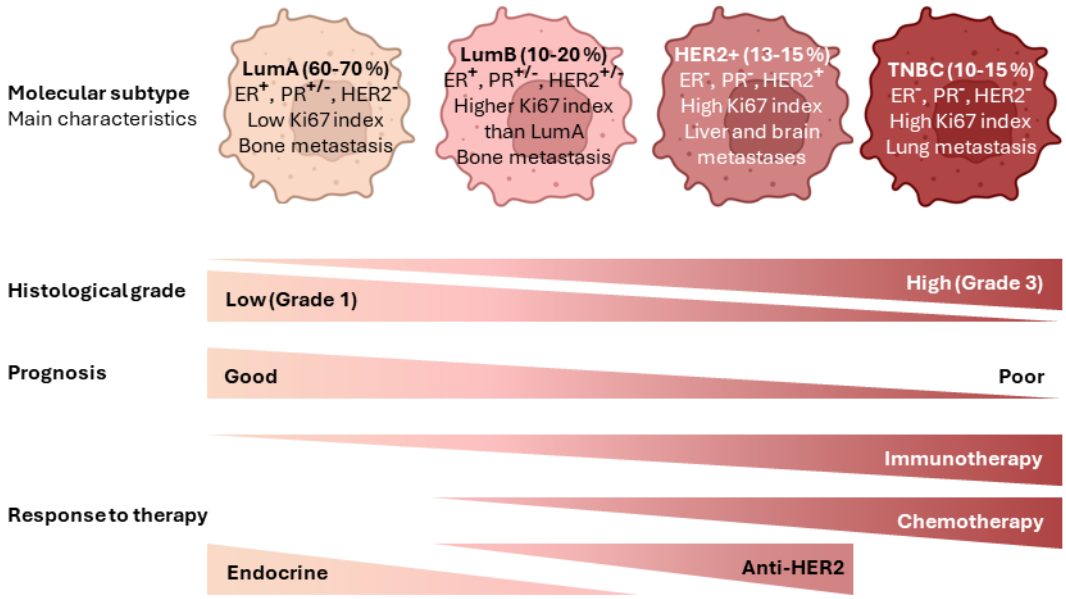

FIGURE I1. BC intrinsic molecular subtypes identified by gene expression profiling. The scheme represents the most relevant characteristic of each subtype. LumA: luminal A. LumB: luminal B. HER2+: HER2-enriched. TNBC: triple negative BC.

LUMINAL A AND B TUMOURS

Luminal tumours, the most common subtype of BC (70-80 %), are characterized by the expression of genes typical of luminal breast epithelial cells (such as cytokeratin 8 and 18) as well as oestrogen receptor (ER). Luminal A tumours are low grade, with low proliferative rates (low Ki67 index) and low-risk gene expression signature, thus having the best prognosis among all intrinsic subtypes. Luminal B tumours, on the contrary, express lower levels of hormone receptors [ER and progesterone receptor (PR)], variable expression of HER2-related genes and exhibit higher proliferative rates and tumour grade, therefore having a worse prognosis (Harbeck et al., 2019).

Treatment of luminal tumours is based on blocking ER signalling through hormonal or endocrine therapy, which includes i) selective ER modulators, such as tamoxifen, ii) selective ER downregulators, such as fulvestrant iii) aromatase inhibitors—the main enzyme responsible for oestrogen synthesis—such as letrozole, and iv) ovarian function suppressors, such as leuprolide. Luminal B tumours are also candidates for additional chemotherapy and, in the case of HER2-expressing tumours, anti-HER2 targeted therapies (discussed in HER2-enriched tumours) (Da Silva et al., 2023).

HER2-ENRICHED TUMOURS

HER2-enriched tumours (13-15 %) are characterised by the overexpression of human epidermal growth factor receptor 2 (HER2), a tyrosine-kinase receptor belonging to the epidermal growth factor receptor (EGFR) family, and by low or null expression of ER and PR. These receptors, after ligand-induced dimerization, control signalling pathways involved in proliferation, survival and differentiation, so their overexpression would provide these cells a growth advantage (Ménard et al., 2003). These tumours display a high Ki67 index and histological grade, making them more aggressive than luminal tumours.

The prognosis of patients with HER2-positive tumours has significantly improved with the introduction of anti-HER2 therapies, including monoclonal antibodies targeting the extracellular domain involved in ligand binding and receptor dimerization—and thus activation—such as trastuzumab and pertuzumab; small tyrosine kinase inhibitors, such as lapatinib; or antibody-drug conjugates that combine an antibody with a cytotoxic agent, like TDM-1 or trastuzumab emtansine (X. Cheng, 2024). Conversely, patients with HER2-enriched tumours respond poorly to hormone therapy, probably due to the inverse relationship between ER expression and HER2 overexpression (Ménard et al., 2003).

BASAL-LIKE TUMOURS

Basal-like tumours (10-15 %), often referred to as triple negative breast (TNB) tumours, are defined by immunohistochemical lack of expression of ER, PR and HER2. Their aggressive behaviour (high histological grade, increased risk of recurrence and propensity for metastasis), along with the lack of selective therapeutic targets, makes them the BC subtype with the poorest prognosis (Harbeck et al., 2019).

TNBC is a heterogeneous group of diseases whose biology is poorly understood. In 2011, Lehmann and collaborators identified 6 molecular subtypes of TNBC, which they later refined to 4 transcriptional subtypes (basal-like 1, basal-like 2, luminal androgen receptor and mesenchymal) (Lehmann et al., 2016). On the other hand, Burstein's group identified 4 TNBC subtypes: Luminal/Androgen Receptor, Mesenchymal, Basal-Like Immune Suppressed and Basal-Like Immune Activated (Burstein et al., 2015). Despite these differences, both studies concur that TNBC subtypes exhibit biological distinctions, and their characterisation provides significant value for clinical decision-making.

The management of TNBC is challenging due to the lack of specific molecular targets, and the standard multimodal approach based on the combination of chemotherapeutic agents (in both neo- and adjuvant settings) with surgery and radiotherapy has not significantly changed the overall survival rate (less than 50 % of patients achieved a pathological complete response (pCR) in 2020) (J. S. Lee et al., 2020). Advances in understanding TNBC biology have led to the identification of specific biomarkers that have become potential therapeutic targets. For example, patients with germline BRCA1/2 mutations develop tumours with an impaired response to DNA double-strand breaks, which makes them particularly sensitive to poly(ADP-ribose) polymerase inhibition. Inhibitors of this enzyme, such as olaparib and talazoparib, have been approved by the Food and Drug Administration (FDA) first for metastatic disease and subsequently for high-risk early-stage disease (Zagami & Carey, 2022).

However, the studies of Lehmann, Burstein and colleagues also highlight the importance of other non-cancer cells, particularly those of the immune system, in the pathogenesis of BC, therefore suggesting that some patients may benefit from therapeutic strategies focused on reinvigorating the antitumour response of the immune system [e.g., immune checkpoint inhibitors (ICIs), **Box 2**]. Compared to other solid tumours, such as melanoma or lung cancer, BC is generally considered an immune cold tumour, meaning it typically exhibits low levels of immune cell infiltration and limited activation of immune pathways. This immune quiescence is attributed to factors such as the relatively low tumour mutation burden (TMB) and the presence of immunosuppressive elements, which together result in poor recognition and targeting by the immune system (Gatti-Mays et al., 2019). However,

compared to the other BC subtypes, TNBC stands out as the most immunogenic due to its genomic instability and its relatively high TMB, which increase the likelihood of generating novel immunogenic peptides (neoantigens). This, coupled with the elevated levels of programmed death ligand 1 (PD-L1), may explain the higher proportion of tumour-infiltrating lymphocytes (TILs) observed in these tumours (Abdou et al., 2022), which are also indicative of better outcomes (Loi et al., 2019). In line with these immunogenomic features, all of which are indicative of a more active immune microenvironment, **immunotherapy** has emerged as a promising therapeutic option for these patients.

Box 2. Physiopathology of immune checkpoints

PD-1 (programmed cell death protein 1) and its ligands are commonly referred to as immune checkpoints due to their central role in regulating immune homeostasis and tolerance, thereby preventing autoimmunity. PD-1 is a receptor predominantly expressed on activated T cells, but can also be found on B cells, natural killer (NK) cells, myeloid cells, and other immune cell subsets. PD-L1, one of the main PD-1 ligands, is ubiquitously expressed on immune cells as well as non-hematopoietic cells and is frequently upregulated on tumour cells.

The regulation and function of PD-1 in T cells has been extensively studied. Under basal conditions, PD-1 expression is minimal but is rapidly induced following T cell activation, serving as a critical feedback mechanism to maintain the delicate balance between effective immune responses and the prevention of excessive activation that could lead to tissue damage. Engagement of PD-1 by PD-L1 attenuates T cell receptor (TCR) signalling, inhibiting T cell proliferation, activation and cytokine production. It can also induce apoptosis, thereby controlling the magnitude and duration of immune responses.

In cancer, tumour cells exploit this signalling pathway to evade the antitumour immune response. In response to inflammatory signals such as interferon-γ (IFN-γ), cancer cells upregulate PD-L1, which interacts with PD-1 on tumour-infiltrating lymphocytes leading to T cell dysfunction, exhaustion and impaired cytotoxic activity, allowing tumour cells to evade immunosurveillance.

In summary, the PD-1/PD-L1 axis plays a dual role restraining immune activation to prevent autoimmunity and tissue damage, while also enabling tumour immune evasion. Targeting either component with immune checkpoint inhibitors (i.e., monoclonal antibodies) can restore T cell function and promote durable antitumour responses, particularly in solid tumours (**Figure B2)** (Alsaab et al., 2017; R.-Y. Chen et al., 2023; Gao et al., 2024).

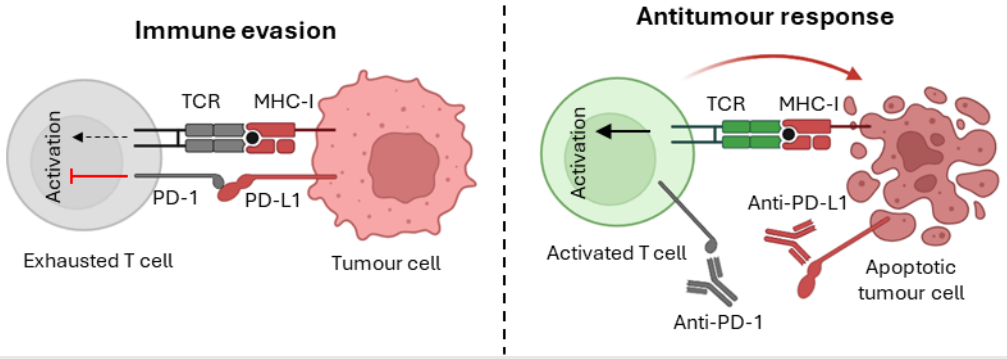

Figure B2. Immune checkpoint blockade in cancer. (Left) The antigen presented on a major histocompatibility complex class I (MHC-I) molecule by a tumour cell is recognised by the TCR expressed on a tumour-infiltrating T cell. This interaction triggers the activation of the effector functions of the T cell, ultimately leading to the targeted killing of the cancer cell. However, the engagement of PD-1 expressed on the surface of the activated T cell with its ligand on tumour cells attenuates TCR signalling leading to T cell anergy and facilitating immune escape. (Right) Therapeutic monoclonal antibodies targeting either PD-1 or PD-L1 disrupt this inhibitory axis, restoring T cell activity and enabling its antitumoural response.

Immunotherapy for TNBC

Immunotherapy for BC was introduced in 2019 with the accelerated FDA approval of atezolizumab (an anti-PD-L1 monoclonal antibody) combined with nab-paclitaxel as a first-line treatment for PD-L1-positive (PD-L1+), metastatic TNBC (mTNBC) based on progression-free survival (PFS) benefits demonstrated in the IMpassion130 trial (P. Schmid et al., 2018). However, this combination was withdrawn as the IMpassion131 trial failed to confirm significant improvement in either overall survival (OS) and PFS compared to chemotherapy alone in PD-L1+ patients (Miles et al., 2021). Alternatively, in November 2020 the FDA granted accelerated approval to pembrolizumab (an anti-PD-1 monoclonal antibody) combined with chemotherapy to PD-L1+ locally recurrent unresectable or mTNBC based on KEYNOTE-355 trial data demonstrating significant PFS improvement in this population (Cortes et al., 2022). Pembrolizumab also received regulatory approval in 2021 for high-risk, early-stage TNBC in combination with chemotherapy in the neoadjuvant setting and as monotherapy adjuvant treatment. This approval was based on significant benefit in both pCR and event-free survival demonstrated in the phase III KEYNOTE-522 trial (P. Schmid et al., 2020).

ICIs administered as monotherapy have demonstrated durable objective responses in a subset of patients across multiple clinical trials [reviewed in (Howard et al., 2022)], though with modest response rates specially in metastatic trials probably because metastatic lesions are immunologically more inert than their corresponding primary tumour (Szekely et al., 2018). These findings support the idea that ICI monotherapy may demonstrate superior clinical efficacy in early-stage TNBC compared to advanced disease settings. The BELLINI trial exemplifies this approach, demonstrating that ICI without chemotherapy elicits clinically meaningful responses in early-stage TNBC, particularly within PD-L1+ and TILs-high subgroups (Nederlof et al., 2024).

In terms of biomarkers of response, PD-L1 expression has already been incorporated in the clinical management of TNBC. However, several limitations challenge its predictive value, including i) spatiotemporal heterogeneity in its expression (e.g., early- vs late-stage disease, primary tumour vs metastatic sites, or chemotherapy-induced expression) or ii) the absence of standardised assays for its assessment (e.g., antibody variability, inconsistent cut-off thresholds and subjective staining interpretation) (L. Li et al., 2023; X. Wang et al., 2023).

TILs are clinically validated prognosis biomarkers for TNBC with robust evidence linking high TILs to improved outcomes [OS and disease-free survival (DFS)] in retrospective-prospective studies (S. Adams et al., 2014; Ali et al., 2014; Loi et al., 2013; J. H. Park et al., 2019). There is also emerging evidence supporting its predictive value in response to ICIs: KEYNOTE-086 (Loi et al., 2023) and KEYNOTE-119 (Loi et al., 2020) clinical trials concluded that high baseline stromal TILs may identify patients more likely to respond to pembrolizumab. However, there are also contradictory findings as the GeparNuevo study (Loibl et al., 2022) showed no improvement in pCR rates between the placebo and anti-PD-1 arms. Consequently, the predictive utility of TILs for ICI efficacy in TNBC remains inconclusive and further independent prospective studies are required to clarify their clinical applicability.

THE METASTATIC DISEASE

Despite significant advances in early detection and therapeutic strategies that have markedly improved clinical outcomes, 20-30 % of patients diagnosed with early-stage BC will develop distant metastasis, which remains the leading cause of cancer-related mortality, accounting for approximately 90 % of patient deaths (Bonotto et al., 2014).

The metastatic process refers to the multi-step mechanism by which a primary tumour disseminates to distant anatomical sites, encompassing: i) the escape of disseminated tumour cells (DTCs) from the primary site; ii) local invasion into adjacent tissues; iii) intravasation into circulation and subsequent survival; iv) extravasation into distant organs; and v) metastatic seeding at secondary sites, where bidirectional crosstalk between DTCs and the microenvironment critically determines cellular fate—including cell death, dormancy or metastasis outgrowth (**Figure I2**). In addition to the linear metastasis model, which suggests that only advanced neoplasms can give rise to distant metastasis, BC preferentially follows the parallel model, in which DTCs spread early during tumourigenesis acquiring simultaneously, yet independently, metastatic traits at secondary sites, leading to genomic divergence from the primary tumour (Riggio et al., 2021).

Regardless of the dissemination model, all BC subtypes can metastasise, with bone being the most common metastatic site, followed by the liver and brain. However, the likelihood of metastasis to specific organs (i.e., organotropism) varies significantly by molecular subtype: luminal A/B tumours are prone to bone metastasis, HER2-enriched subtypes show high rates of liver and brain metastases, and TNBC primarily presents with lung metastases (W. Chen, Hoffmann, et al., 2018). In 1889, Stephen Paget proposed the “seed and soil” hypothesis to explain that this organ-specific colonization is not a random process, but rather the result of the compatibility between DTCs (the seed) and the growth-permissive conditions in distant organs (the soil) (Paget, 1889). Muller and colleagues validated this concept by demonstrating that C-X-C chemokine receptor type 4 (CXCR4) – stromal cell-derived factor-1 (SDF1, also known as CXCL12) axis facilitate organ-specific metastasis as the receptor is upregulated by BC cells and its sole known ligand is highly expressed in organs representing the primary metastatic destinations for BC, such as bone, liver and lung (Müller et al., 2001).

Paget’s hypothesis significantly evolved with the idea that primary tumours actively prime distant organ microenvironment for metastasis through the formation of a pre-metastatic niche (PMN). This priming is initiated by the secretion of tumour-derived soluble factors and exosomes from the primary tumour, which target both secondary organs and the bone marrow. In 2005, Lyden and colleagues demonstrated that vascular endothelial growth factor A (VEGF-A) secreted by primary tumours recruits bone marrow-derived VEGF receptor-1/very late antigen-4 (VEGFR-1/VLA-4)-positive hematopoietic progenitor cells to fibronectin-rich lung regions, where they secrete matrix metalloproteinase 9 (MMP9) to degrade the extracellular matrix (ECM) and facilitate DTCs colonisation (Kaplan et al., 2005). Building on this, Xiang and collaborators revealed that BC-derived exosomes could drive bone marrow-myeloid cells differentiation into myeloid-derived suppressor cells (MDSCs) (X. Xiang et al., 2009), which accumulate in the lung via hypoxic BC-secreted lysyl oxidase (LOX)-mediated collagen IV crosslinking (Erler et al., 2009). These MDSCs suppress antitumour T cells through arginase-1 and reactive oxygen species (ROS) production.

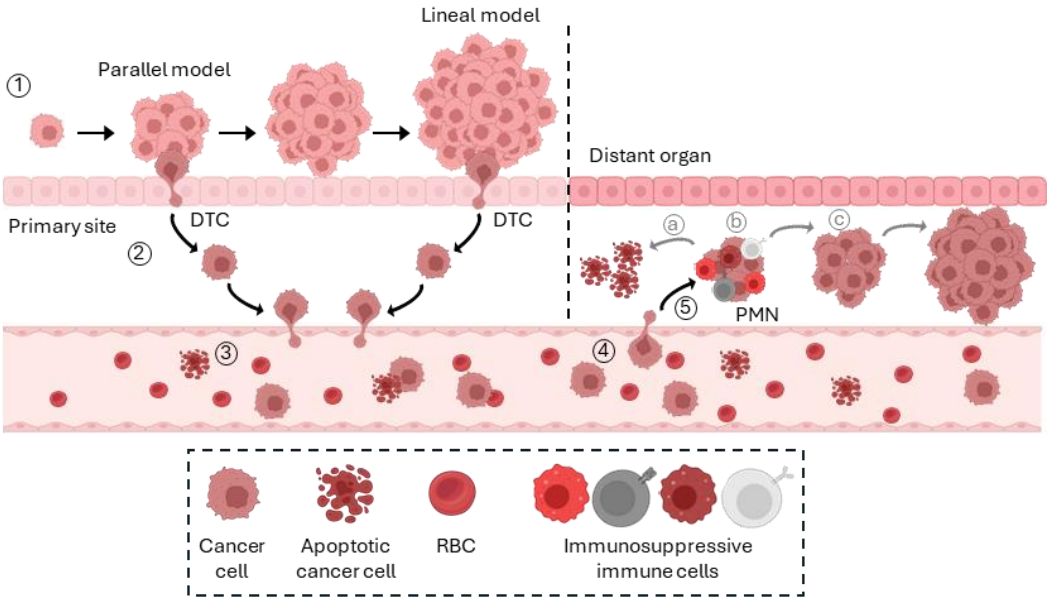

Figure I2. Metastasis cascade. (1) Disseminated tumour cells (DTCs) escape from the primary tumour at early (parallel model) or late (linear model) stages of tumourigenesis, (2) invade adjacent tissues and (3) intravasate into circulation. If they survive, they (4) extravasate into distant organs and (5) the metastatic seeding occurs. DTC fate is now determined by its bidirectional crosstalk with the tumour microenvironment and may result in (a) cell death, (b) tumour dormancy, or (c) metastatic outgrowth. PMN: pre-metastatic niche; RBC: red blood cell.

A pivotal tumour-derived chemokine in PMN formation is C-C motif ligand 2 (CCL2), which preferentially recruits C-C chemokine receptor type 2 (CCR2)-positive inflammatory monocytes to promote DTC extravasation and survival via VEGF and interleukin-6 (IL-6) and fibronectin production, respectively (B.-Z. Qian et al., 2011; H. Zhang et al., 2018). CCL2 also recruits dysfunctional NK cells with impaired cytotoxic capacity, therefore unable to eliminate incoming DTCs (Sceneay et al., 2012). Additionally, PMN lung expresses thymus and activation regulated chemokine (TARC, also known as CCL17) and macrophage-derived chemokine (MDC, also known as CCL22), which attract CCR4-positive regulatory T cells (Tregs) and tumour cells, further supporting metastatic seeding (Olkhanud et al., 2009).

Collectively, these changes involve a systemic, tumour-mediated immune remodelling that establishes an immunosuppressive microenvironment permissive for metastasis.

THE TUMOUR MICROENVIRONMENT

The reductionist concept of cancer as a group of transformed malignant cells that proliferate uncontrollably due to the accumulation of genetic mutations does not fully explain the complexity of this disease in terms of progression and response to therapy. Over the past few decades, there has been a shift towards a more holistic perspective, whereby cancer is no longer regarded as an isolated cluster of tumour cells, but rather as a complex and dynamic ecosystem referred to as the tumour microenvironment (TME) (De Visser & Joyce, 2023).

Although the composition and functional state of the TME exhibit significant interpatient variability—even within the same cancer type—its core structure comprises non-cellular (such as ECM and soluble signalling factors) and cellular components (including endothelial cells, fibroblast and immune cells) that will be discussed along this section (**Figure I3**). As the heart of the TME, cancer cells actively remodel their surrounding by altering vasculature, reconfiguring ECM architecture and reprogramming host stromal cells to adopt pro-tumourigenic functions. These adaptations create a self-reinforcing ecosystem that supports tumour growth and metastasis, as well as therapy resistance. Understanding the mechanisms underlying these changes and their subsequent implications is critical for enabling clinicians to effectively stratify patients, thereby facilitating oncological decision-making.

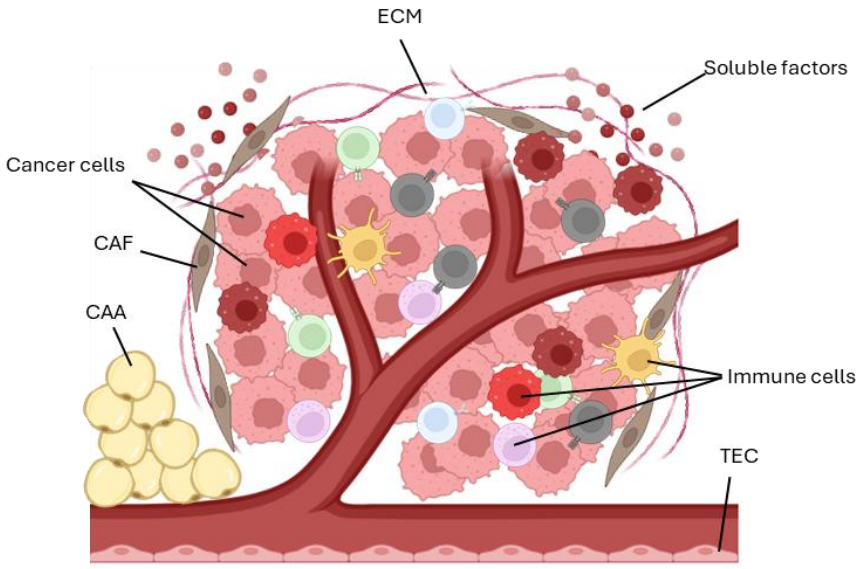

Figure I3. Schematic representation of the principal components of the TME. The core structure comprises both cellular and non-cellular elements that interact with cancer cells creating an ecosystem that promotes tumour progression. CAA: cancer-associated adipocyte; CAF: cancer-associated fibroblast; ECM: extracellular matrix; TEC: tumour-associated endothelial cell.

NON-CELLULAR COMPONENTS OF THE TUMOUR MICROENVIRONMENT

THE EXTRACELLULAR MATRIX

The ECM is a three-dimensional network of proteins, proteoglycans, glycoproteins and polysaccharides that provides structural and biochemical support to tissues. Beyond its mechanical role, the ECM facilitates intercellular communication by sequestering secreted

molecules (e.g., growth factors or cytokines) and serving as a substrate for cell adhesion, migration and signalling (Winkler et al., 2020) (**Figure I4**).

In BC, the ECM undergoes dynamic remodelling that alters its biophysical and biochemical properties through different mechanisms: i) ECM deposition (i.e., changes in composition), exemplified by elevated levels of small hyaluronan oligosaccharides, which are associated with poor prognosis due to their ability to regulate pro-tumourigenic signalling cascades; ii) post-translational modifications and organisation, such as LOX-mediated collagen crosslinking, which makes ECM stiffer and, in addition to activate survival and migration signalling pathways, reduces drug penetrance and excludes cytotoxic T cells from the TME (favouring immune evasion); and iii) ECM degradation, notably involving MMPs, upregulated in transformed breast tissue) that facilitate tumour progression by degrading the basement membrane (enabling tumour cells to invade adjacent stroma) or releasing growth-promoting [e.g., epidermal growth factor (EGF), amphiregulin] and anti-apoptotic factors [e.g., transforming growth factor β (TGF-β)] (Radisky, 2015; Winkler et al., 2020).

SOLUBLE SIGNALLING FACTORS

Soluble mediators—including cytokines, chemokines and growth factors—serve as pivotal regulators of the TME dynamics by orchestrating intercellular communication (**Figure I4**). Through their dual influence on tumour and non-malignant stromal cells, they finely regulate the equilibrium between tumour-promoting and tumour-suppressive mechanisms, exemplified by their roles in immune modulation, cellular proliferation and angiogenesis (De Visser & Joyce, 2023).

TGF-β is the most extensively studied cytokine in BC, exhibiting stage-dependent dual roles. During early tumourigenesis, it acts as a tumour suppressor by inducing cell cycle arrest and suppressing ER-mediated proliferation, although resistance to its antiproliferative actions is frequently observed in TNBC cell lines. In advanced stages, it promotes tumour progression through direct effects on BC cells (destabilising tumour suppressor proteins such as p53, inducing epithelial-to-mesenchymal transition (EMT), and driving therapy resistance) and stromal modulation (converting mammary fibroblasts into cancer-associated fibroblasts (CAFs), recruiting MDSCs, and suppressing NK and antigen-specific T cell cytotoxicity) (W. Chen, Qin, et al., 2018; Zu et al., 2012).

Interferon-gamma (IFN-γ) is a pleiotropic cytokine whose bioavailability in the TME dictates its functional duality: high levels drive antitumour effects (such as cancer cell apoptosis, enhanced tumour antigenicity, and blood vessel destruction), whereas low concentrations promote pro-tumoural activity—as observed in stage III breast tumours (Yi et al., 2024)—including enhanced metastatic potential, cancer stem cell expansion (Beziaud et al., 2023), and immune evasion through PD-L1, nitric oxide synthase 2 and cyclooxygenase-2 (COX2) overexpression on BC cells (Y. S. R. Cheng et al., 2022), coupled with TIL depletion. Therefore, IFN-γ is critical for the success of immunotherapy and high levels correlate with improved responses to ICI as they may reflect functional T cell activity (Jorgovanovic et al., 2020).

CXCL12 is a homeostatic chemokine with a detrimental role in BC through its correlation with metastasis and reduced patient survival. It recruits CXCR4-expressing BC cell, facilitating metastasis and primary tumour growth. Simultaneously, CXCL12 attracts monocytes that differentiate into M2 macrophages—linked to chemotherapy resistance

and relapse (Hughes et al., 2015)—and Tregs (Yan et al., 2011), while excludes cytotoxic T cells from the tumour core by sequestering them in the stroma (Murdamoothoo et al., 2021).

CCL2 is an inflammatory chemokine exhibiting dual roles within the BTME: during early carcinogenesis, it mediates antitumour immunosurveillance (M. Li et al., 2013), whereas in established neoplasms, it drives tumour growth, metastatic dissemination, and therapy resistance, particularly in aggressive subtypes (e.g., HER2-enriched), correlating with reduced relapse-free survival. Its pro-tumoural effects arise from direct CCR2-positive BC cell signalling (enhanced survival and migration), recruitment of CCR2-positive inflammatory monocytes that differentiate into pro-tumoural macrophages, or mobilisation of endothelial precursors to facilitate neovascularisation (X. Chen et al., 2016; D. Li et al., 2020; Yoshimura et al., 2023).

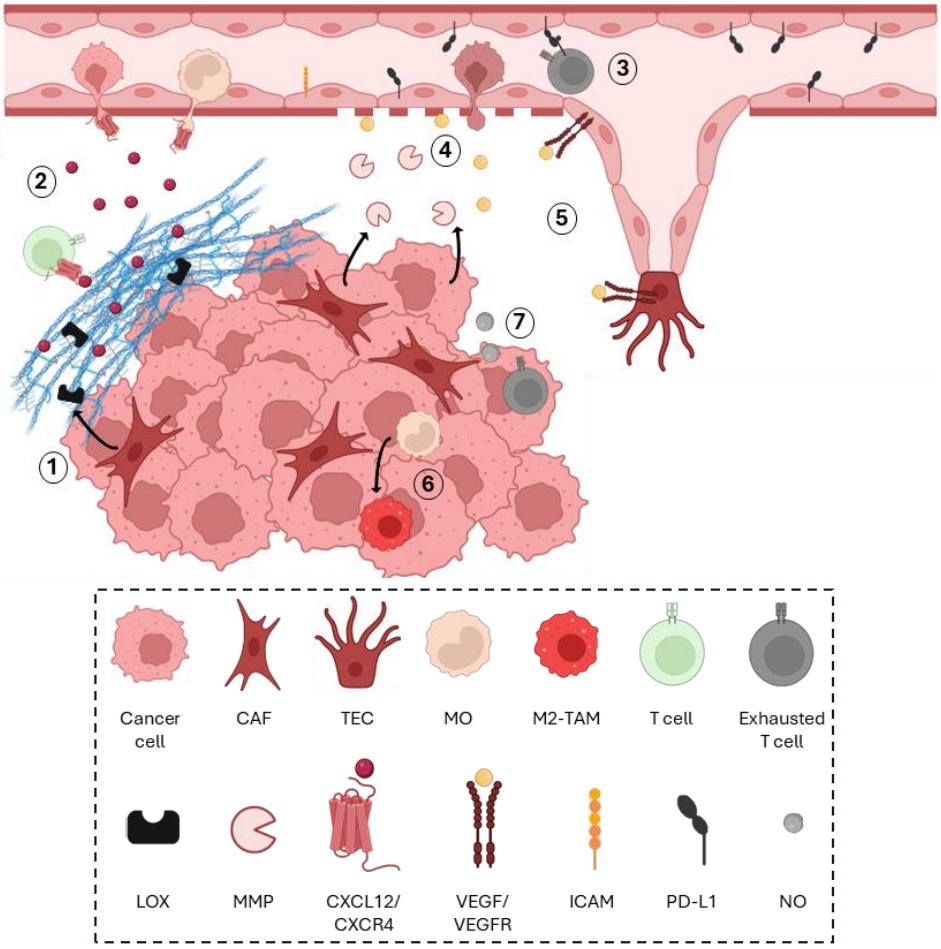

Figure I4. Schematic representation of key interactions among the ECM, soluble factors, CAFs and TECs within the TME. (1) CAFs increase ECM stiffness via LOX-mediated crosslinking of collagen, which enhances cancer cell migration, reduces drug penetrance, and excludes cytotoxic T cells. (2) ECM-bound CXCL12 establishes stable chemotactic gradients that induce the migration of CXCR4-positive cells, including cancer cells, monocytes (MO) and T cells. (3) TECs downregulate leukocyte adhesion molecules (ICAM) and overexpress PD-L1, leading to T cell dysfunction and reduced extravasation. (4) Cancer cells and CAFs secrete MMPs, which degrade the basement membrane – enabling tumour cells to invade adjacent stroma – and release pro-angiogenic factors that (5) promote TEC migration and proliferation, resulting in abnormal vessel formation. (6) CAFs polarize MO toward M2-TAM and (7) produce NO, contributing to T cell suppression.

CELLULAR COMPONENTS OF THE TUMOUR MICROENVIRONMENT

TUMOUR-ASSOCIATED ENDOTHELIAL CELLS

Endothelial cells (ECs) build up the inner layer of blood and lymphatic vessels and are involved in numerous processes including vascular hemodynamic and permeability, cell extravasation and angiogenesis. Tumour-associated ECs (TECs) exhibit distinct functional, phenotypic and gene expression profiles compared to normal ECs, enabling them to promote cancer progression (Rodríguez-Bejarano et al., 2024).

Upon tumour growth, hypoxia activates hypoxia-inducible factors, triggering the release of pro-angiogenic factors like VEGF-A (linked to poor BC prognosis) that drive TEC proliferation, migration and assembly. Due to excessive signalling, structurally abnormal vessels—immature and lacking proper cell-to-cell connections—are generated, therefore contributing to cancer cell extravasation (Leone et al., 2024). TECs also display high plasticity, undergoing endothelial-to-mesenchymal transition via hypoxia, TGF-β and microRNAs to transdifferentiate into mesenchymal cells—primarily CAFs (see below)—thereby enhancing stromal remodelling and pro-tumourigenic signalling in the breast TME (BTME). Additionally, TECs exert immunomodulatory effects by downregulating leukocyte adhesion molecules, impairing cell migration, and overexpressing inhibitory molecules such as PD-L1, which not only suppresses T cell killing activity, but also establishes a selective barrier that reduces infiltration of cytotoxic T cells while promoting Treg accumulation (**Figure I4**), thus fostering an immunosuppressive TME that supports tumour progression (J. Fang et al., 2023).

CANCER-ASSOCIATED FIBROBLASTS

Under physiological conditions, fibroblasts participate in the wound healing response by undergoing reversible activation to repair damaged tissue. In cancer, however, these cells acquire a perpetually activated state, which drives tumour progression through sustained ECM remodelling—altering its composition and increasing stiffness—and pro-angiogenic signalling (e.g., VEGF-mediated EC recruitment) (Rodríguez-Bejarano et al., 2024). Additionally, they confer chemotherapy resistance by inducing and maintaining a stem-like phenotype of TNBC cells (Cazet et al., 2018). Furthermore, CAFs promote immunosuppression through multiple mechanisms, including direct suppression of T cell activity via nitric oxide (NO) production (Cremasco et al., 2018); recruitment and polarisation of immunosuppressive myeloid cells (e.g., pro-tumoural M2 macrophages) (Cohen et al., 2017); accumulation and activation of Tregs (Costa et al., 2018) or by skewing the balance of helper T cell subsets to favour Th2 over Th1 responses (i.e., favouring pro- against antitumour immunity) (**Figure I4**) (Liao et al., 2009).

CAFs are the most prominent stromal type in the BTME and represent a heterogeneous population arising from multiple sources—including the expansion of local tissue-resident fibroblasts or the conversion of other cell types such as bone marrow-derived mesenchymal stem cells, adipocytes, or cancer cells undergoing EMT—which may explain their functional heterogeneity. Importantly, these CAFs subsets have prognostic value by association to metastatic disease and may also predict treatment resistance (Bartoschek et al., 2018; Bochet et al., 2013; Raz et al., 2018).

CANCER-ASSOCIATED ADIPOCYTES

A significant percentage of the breast tissue consists of adipocytes, specialised cells that regulate energy balance through lipid storage and play a critical role in synthesising and secreting hormones (e.g., oestrogen) and growth factors (e.g., VEGF), thereby controlling mammary gland homeostasis while contributing to BC progression, particularly in obesity—a well-established BC risk factor. Tumour cells induce the differentiation of mature adipocytes into cancer-associated adipocytes that drive tumour progression by remodelling the ECM through secretion of MMPs, thus facilitating BC cell invasion; fuelling cancer metabolism via lipolysis-driven fatty acid release; secreting adipokines (e.g., leptin) and pro-metastatic factors (e.g., CCL2, IL-6); and suppressing antitumour immunity through PD-L1 expression and T cell exhaustion (Rodríguez-Bejarano et al., 2024).

IMMUNE CELLS

The immune system is a highly coordinated network of molecular mediators, cells, tissues and organs that orchestrates host defence against pathogens or abnormal cells while preserving self-tolerance. Although considered low-immunogenic relative to other cancer types, immune cells emerge as critical regulators within the BTME—especially in TNBC—where they demonstrate both tumour-promoting and tumour-suppressive actions (**Figure I5**). The immune composition of the BTME, which will be discussed in detail below, significantly varies across BC subtypes, directly influencing tumour progression and treatment efficacy (Denkert et al., 2018; Klopfenstein et al., 2021).

T cells

T cells are adaptive immune cells that perform distinct functions based on their effector differentiation states. Historically, their heterogeneity has not been considered and their clinical relevance in cancer has been oversimplified through stromal TIL assessments—heterogeneous mononuclear cell population encompassing both T and B cells—quantified via haematoxylin and eosin-stained tumour sections as standardised by The International TILs Working Group (Salgado et al., 2015).

BC subtypes exhibit variable TIL infiltration, with TNBC and HER2-enriched tumours demonstrating significantly higher lymphocyte densities compared to luminal subtypes (Dieci et al., 2018). While elevated stromal TILs generally correlate with improved prognosis and response to therapy (S. Adams et al., 2015; Kurozumi et al., 2019; Stanton & Disis, 2016), studies in ER-positive patients reveal paradoxical associations with adverse outcomes (Kurozumi et al., 2019), underscoring that lymphocytic infiltration magnitude alone does not fully dictate disease progression. Emerging evidence suggests that lymphocyte phenotype may also influence clinical outcome (Stanton & Disis, 2016), indicating that further characterisation of functional T cell heterogeneity in the TME is needed (**Figure I5**).

Helper T cells

CD4 T cells, commonly termed helper T cells, mediate host defence by orchestrating immune responses through their interactions with effector cells

including cytotoxic T cells and B cells. These cells differentiate into functionally distinct subtypes in response to context-dependent signals, thus having different roles in tumour immunology.

Type 1 helper T cells (Th1) demonstrate canonical antitumour activity through the secretion of soluble factors (like IFN-γ or IL-12) that enhance antigen-presenting cells (APCs) capacity to prime CD8 T cell responses, activate macrophage tumouricidal activity (DeNardo & Coussens, 2007), or directly induce senescence in cancer cells (Braumüller et al., 2013). In contrast, type 2 helper T cells (Th2) typically secrete cytokines associated with anti-inflammatory responses (such as IL-4 and IL-10) that may promote tumour progression by polarising macrophages towards protumour phenotypes and inducing T cell disfunction (DeNardo & Coussens, 2007). However, emerging evidence suggests that Th2 cells can also mediate antitumour effects by inducing the terminal differentiation of BC cells (Boieri et al., 2022), underscoring the context-dependent nature of its function.

Clinical evaluation of human BC samples reveals that a Th1-enriched breast tumour immune microenvironment (BTIME) correlates with improved prognosis, while elevated Th2/Th1 ratios associate with reduced OS (Zareinejad et al., 2023). Longitudinal studies demonstrate that Th1 responses diminish upon tumour progression in parallel with an expansion of Th2 activity, a shift linked to metastatic dissemination (Faghih et al., 2014). Furthermore, Th1 response attenuation correlates with residual disease post-treatment and increase recurrence risk (Fracol et al., 2017).

Cytotoxic T cells

CD8 T cells, also known as cytotoxic T cells (CTLs), constitute a critical component of antitumour immunity through their ability to recognise and eliminate malignant cells. Recent advances have identified effector CTL subsets mirroring Th differentiation patterns, with IFN-γ-secreting CTL representing the most extensively characterised subset (St. Paul & Ohashi, 2020). Following APC-mediated priming, CTLs specifically recognise tumour antigens presented on major histocompatibility complex (MHC) class I molecules via T cell receptor (TCR) engagement. This interaction triggers cytotoxic mechanisms including perforin-granzyme exocytosis and Fas cell surface receptor (Fas)-Fas ligand (FasL) signalling pathways, ultimately inducing target cell apoptosis. Concurrently, CTL-derived IFN-γ contributes to establishing an immunostimulatory TIME (Moura et al., 2025).

Several studies demonstrate significant associations between robust CTL infiltration and improved clinical outcomes in BC, including enhanced therapy response rates and prolonged survival (Mahmoud et al., 2011; Oshi, Asaoka, et al., 2020; Sun et al., 2022; Triulzi et al., 2015), especially when CTLs localise proximal to malignant cells (H. Liang et al., 2025). However, tumours employ multiple immune evasion strategies, including MHC class I downregulation (Y. Fang et al., 2021) and subversion of physiological tolerance mechanisms. Chronic antigen exposure promotes sustained expression of immune checkpoint molecules such as PD-1, which engages tumour-expressed PD-L1 to drive T cell exhaustion—a

dysfunctional state characterised by impaired cytotoxic function. This exhausted phenotype correlates with poor clinical outcomes and reduced therapeutic responsiveness (Bense et al., 2017; Feng et al., 2024), making T cell reinvigoration strategies a key focus in cancer immunology research.

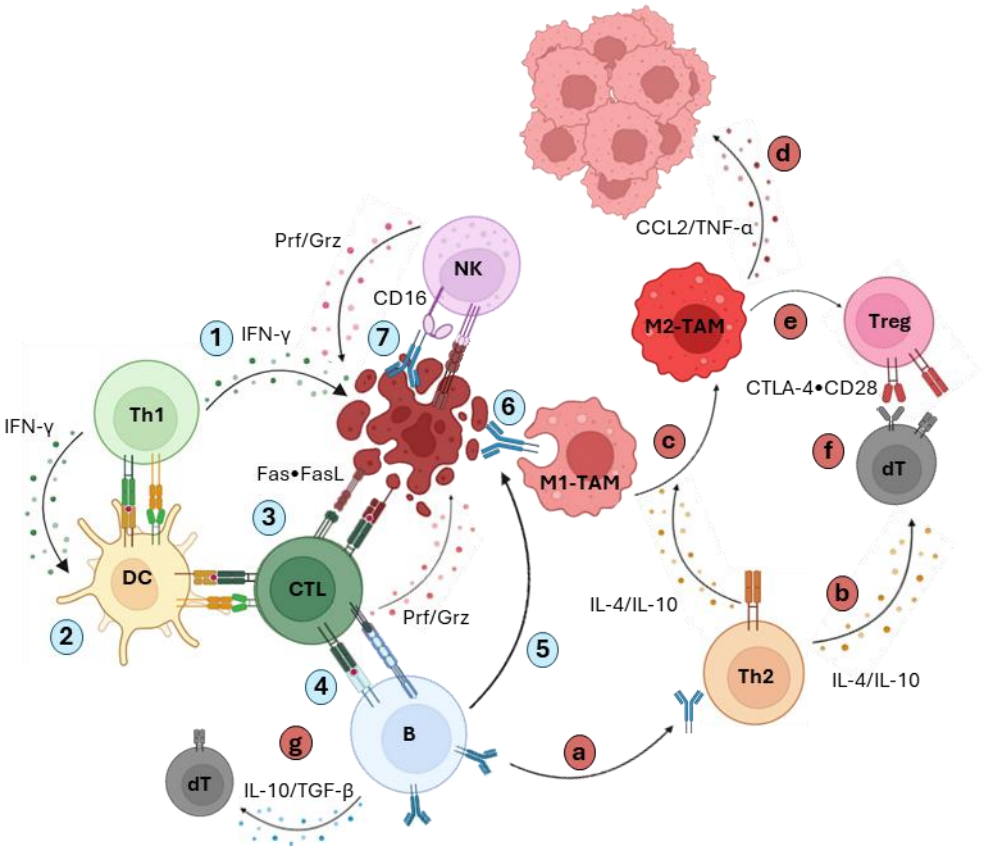

Figure I5. Interactions between immune and tumour cells within the TIME. Blue circles indicate antitumour functions, whereas red circles denote pro-tumoural actions. Th1 cells secrete IFN-γ, which may induce cancer cell senescence (1) or enhance DC capacity to prime CTL responses (2), culminating in cancer cell death via cytolytic granule release (Prf/Grz) or ligand-induced apoptosis (Fas-FasL) (3). (4) Memory B cells may function as antigen-presenting cells (APC), thereby promoting T cell activation; while plasma cells produce antibodies (5) that trigger antibody-dependent cellular phagocytosis (ADCP) mediated by macrophages (6), or antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells (7). These antibodies may also favour the differentiation of Th2 cells (a), which secrete IL-4 and IL-10, leading to T cell dysfunction (b) or the polarisation of M1-TAM towards the M2 phenotype (c). The latter secrete factors that promote tumour cell proliferation and survival (d) and induce the differentiation of Tregs (e), which immunosuppress effector T cells via CTLA-4/CD28 interaction (f). Finally, regulatory B cells suppress T cell responses through the release of immunosuppressive cytokines (g). CTL: cytotoxic T cell; DC: dendritic cell; dT: dysfunctional T cell; NK: Natural killer cells; TAM: tumour-associated macrophage; Prf/Grz: Perforin/Granzyme; Treg: regulatory T cell.

Regulatory T cells

Tregs constitute a specialised CD4 T lymphocyte subset defined by constitutive expression of the transcription factor Forkhead box protein 3 (FOXP3) and immunosuppressive functions critical for maintaining self-tolerance, including

cytokine-mediated suppression (e.g., TGF-β and IL-10), metabolic disruption (e.g., IL-2 sequestration and adenosine generation), APC modulation and direct T cell inhibition via cytotoxic T-lymphocyte antigen 4 (CTLA-4)/CD28 pathway interference (Grover et al., 2021).

Multiple clinical studies associate elevated Treg infiltration with adverse outcomes and diminished therapeutic efficacy (Bates et al., 2006; Jamiyan et al., 2020; Stenström et al., 2021), while others show the opposite (L. Zhang et al., 2019). Notably, Treg accumulation frequently coincides with increased CD8 T cell infiltration (R. Chan et al., 2024; S. Liu et al., 2014), and this paradoxical association may reflect the dominant antitumour effects of CTLs in treatment-responsive microenvironments. Nevertheless, the precise prognostic significance of Tregs in BC remains contentious and requires further characterisation.

B cells

B cells are lymphoid linage cells integral to humoral immunity through antibody production. Beyond this canonical role, they exhibit antigen-presenting capabilities and modulate immune responses through cytokine secretion. B cell ontogeny initiates in the bone marrow with sequential immunoglobulin gene rearrangements, culminating in B cell receptor expression on immature B cells. Following negative selection to eliminate autoreactive clones, mature B cells migrate to secondary lymphoid organs where terminal differentiation into either memory B cell or CD138-positive antibody-secreting plasma cells occurs. In microenvironments enriched in IL-6, B cell activating factor and proliferation-inducing ligand, immunosuppressive regulatory B cells (Bregs) may also emerge (M. Li et al., 2023).

CXCL13-mediated chemotaxis recruits B cells to the TME, where they either form peripheral aggregates or organise into tertiary lymphoid structures. In BC, these heterotypic clusters—comprising T cells, B cells and dendritic cells (DCs)—are associated with better outcomes (H. J. Lee et al., 2016; B. Wang et al., 2022), particularly when localised near tumour cell islands (Wortman et al., 2021).

The BTIME typically contains naive B cells (~ 10 %), memory B cells (~ 80 %) and plasma cells (~ 20 %). Memory B cell infiltration correlates with improved clinical outcomes and treatment response across multiple investigations (Brown et al., 2014; Kuroda et al., 2021; Mahmoud et al., 2012), likely mediated through their APC function in T cell activation. Conversely, Breg presence portends poorer prognosis through T cell response suppression (Ishigami et al., 2019; Shen et al., 2018). CD138-positive plasma cell infiltration similarly associates with adverse outcomes (Z. M. Mohammed et al., 2012; Z. M. A. Mohammed et al., 2013). While tumour-associated antibodies may engage antibody-dependent cytotoxicity (ADCC), antibody-dependent cellular phagocytosis, and complement-mediated lysis (Q. Li et al., 2011), these autoantibodies can paradoxically demonstrate pro-tumoural activity or indicate Th2-polarised microenvironments (Garaud et al., 2018) (**Figure I5**).

Natural killer cells

NK cells are cytotoxic innate immune cells initially identified for their virus-infected and malignant cell killing ability, while also orchestrating immune responses by cytokine and chemokine secretion. Based on the expression of the CD56 marker, NK cells are broadly categorised into two functional subsets: NK1 $CD56^{dim}$ (exhibiting potent cytotoxic activity), and NK2 $CD56^{bright}$ (specialised in immunomodulation). NK cells mediate target cell destruction through three principal mechanisms: i) direct cytolytic granule release; ii) ligand-induced apoptosis; and iii) Th1/CTL activation via IFN-γ and tumour necrosis factor-α (TNF-α) production. Notably, CD16-expressing NK cells execute ADCC by recognising immunoglobulin-coated targets—a critical therapeutic mechanism in antibody-based cancer therapies (Arianfar et al., 2021) (**Figure I5**).

In BC, several studies consistently demonstrate an elevated proportion of non-cytotoxic NK2 cells both in tumours and peripheral blood (Carrega et al., 2014; Mamessier et al., 2013; Ostapchuk et al., 2015), potentially mediated by CCL2/CCR2 axis-driven recruitment. Moreover, tumour-infiltrating NK cells display an immature, inhibitory and exhausted phenotype marked by upregulated inhibitory receptors and decrease expression of activating receptor and cytotoxic-related molecules (Krneta et al., 2016; Nieto-Velázquez et al., 2016) and may enhance tumour progression through the activation of BC stem cells (Thacker et al., 2023). This impaired function arises from multiple immunosuppressive mechanisms within the TIME: prostaglandin E2—highly expressed in BC—restricts NK function; soluble activating-ligands competitively inhibit target cell recognition; and matrix metalloproteinases (e.g., ADAM17) downregulate CD16 expression, thereby comprising ADCC efficacy (Rodríguez-Bejarano et al., 2024). Independently of their phenotype, higher number of NK cells in BC patients have been associated with better outcomes, although a clear trend was not observed (Nersesian et al., 2021), and response to therapy (Muntasell et al., 2019; C. Zhang et al., 2024).

Macrophages

Macrophages are innate immune cells with remarkable plasticity capable of adopting either classically activated, pro-inflammatory (M1); or alternatively activated, anti-inflammatory (M2) phenotypes, the former primarily implicated in pathogen/cellular phagocytosis and antigen presentation, whereas the latter participating in wound healing and tissue remodelling (Mehta et al., 2021).

In BC, tumour-associated macrophages (TAMs) constitute a substantial proportion of cells within the BTIME, and they are mainly recruited from monocyte reservoirs via chemokine gradients—including CCL2, CCL5 and granulocyte-macrophage colony-stimulating factor (GM-CSF)—generated by cancer cells. Once in the BTIME, they can acquire a functional polarisation to M1-TAMs with inhibitory properties (e.g., reduction of BC cells invasion and proliferation), but they predominantly assume the M2 phenotype, favoured by the hypoxic conditions, thereby contributing to cancer progression (Mehta et al., 2021). Consistently, the presence of M2-TAMs has been linked to unfavourable clinicopathological features in BC, including higher histological grades or Ki67 index (Badr et al., 2023; Gwak et al., 2015), and reduced

response to treatment, including hormonal therapy and chemotherapy (Arole et al., 2021; Xuan et al., 2014). Moreover, and specifically in TNBC, elevated M2-TAMs levels correlate with increase metastatic potential (Z.-Y. Yuan et al., 2014).

TAMs drive oncogenesis through multiple interconnected mechanisms. They secrete bioactive molecules that promote cancer cell survival (such as CCL2 or TNF-α) or enhance their metastatic potential (e.g., CCL18 and CXCL1 via EMT induction and migration promotion in BC cells) (Su et al., 2014; N. Wang et al., 2018). Metastasis can also be favoured by the production of ECM-degrading enzymes (such as MMPs), while angiogenesis is facilitated through the release of pro-angiogenic factors like VEGF (Leek et al., 2000; Lin et al., 2006). Furthermore, M2-TAMs orchestrate immune evasion by promoting Treg differentiation and recruitment (Kos et al., 2022; J. Liu et al., 2011) or by inhibiting T cell activation and function (Kryczek et al., 2006) (**Figure I5**).

Other immune cells

Dendritic cells

DCs are myeloid linage-derived cells that bridge innate and adaptative immunity. Conventional DCs are well-known APCs, pivotal in initiating T cell responses; while plasmacytoid DCs are the major source of type I interferons, which are linked to antitumour activity through direct activation of CD8 T cells and NK cells (Moura et al., 2025). Nonetheless, their role in BC is still controversial, as high levels of tumour-infiltrating plasmacytoid DCs have been associated with both poor prognosis (Treilleux et al., 2004) or improved outcomes (more active microenvironments) (Oshi, Newman, et al., 2020).

Neutrophils

Neutrophils are the most abundant leucocytes in the bloodstream. Owing to their functional plasticity and the relative content of cytokines within the TME, they can adopt both pro- and antitumoural roles (Rodríguez-Bejarano et al., 2024). In BC, elevated numbers of tumour-associated neutrophils correlate with worse prognosis and therapy resistance (Orditura et al., 2016; Wu et al., 2020). Preclinical studies have also demonstrated that these cells exert immunomodulatory actions by directly inhibiting T cell proliferation (Casbon et al., 2015).

Myeloid-derived suppressor cells

MDSCs are immature, dysfunctional innate immune cells that exert immunosuppression through multiple mechanisms, including oxidative stress induction (via NO and ROS production), arginine depletion (leading to T cell cycle arrest), secretion of immunosuppressive factors such as TGF-β and IL-10 (to promote Treg differentiation and impairment of T and NK cell functions), and expression of PD-L1. These cells are recruited to the TME by cytokines and chemokines such as CCL2, CCL5 or IL-6, with evidence in BC suggesting that an

oestrogen-enriched BTME further enhances this recruitment. Their presence strongly correlates with advanced disease stages, poorer overall survival and metastatic progression (Cha & Koo, 2020; Rodríguez-Bejarano et al., 2024).

Natural killer T cells

Natural killer T (NKT) cells are lipid and glycolipid-reactive T lymphocytes co-expressing markers characteristics of NK cells. They are broadly categorised into two principal subsets: type I or invariant NKT cells, which demonstrate potent antitumour activity, and type II NKT cells, typically associated with pro-tumourigenic functions. Preclinical studies in BC mouse models demonstrate that invariant NKT cell activation induces tumour rejection and reduces metastasis rates, mediated through direct cytolysis of cancer cells and/or reprogramming of the BTIME towards an antitumoural phenotype (Gebremeskel et al., 2015; Hix et al., 2011; Teng et al., 2007).

As evidenced, the heterogeneity of the cellular and non-cellular components comprising the BTME significantly influences disease progression, though its prognostic and predictive potential remains controversial. Crucially, individual assessment of a single BTME component is insufficient for comprehensive evaluation; consequently, researchers have sought to establish stromal gene expression signatures to improve clinical outcome prediction. In 2008, Finak and colleagues developed the stroma-derived prognostic predictor, stratifying patients into two categories independently of the clinical subtype: a good-outcome cluster characterised by Th1-like immune response, and a poor-outcome cluster exhibiting markers of hypoxia and angiogenesis (Finak et al., 2008). Subsequently, Winslow and collaborators identified two additional stromal gene signatures with prognostic value independent of clinicopathological features and molecular subtypes: a poor-prognosis signature associated with complement component 1q genes, and a favourable-prognosis signature linked to elevated human leukocyte antigen gene expression (Winslow et al., 2015). Furthermore, Farmer's group generated a 50-gene stromal signature predictive of poor chemotherapy response, underscoring the clinical relevance of stromal heterogeneity in therapeutic resistance (Farmer et al., 2009).

Despite all the advances in this field, further in-depth analysis of the cellular and functional composition of the BTME is still necessary to gain a more precise understanding of its role in BC progression. This will not only enable to identify novel therapeutic targets for the treatment of this disease, but also prognostic and predictive biomarkers of therapy response.

THE ENDOCANNABINOID SYSTEM

As detailed above, the TME is no longer considered as a passive bystander but rather as an active contributor to cancer biology, thereby emerging as a key source of potential biomarkers and therapeutic targets for cancer treatment. In this regard, the endocannabinoid system (ECS)—a complex, widely distributed cellular communication network with immunomodulatory functions and frequently deregulated in cancer—has attracted increasing scientific interest in recent decades within the field of cancer biology and treatment.

COMPONENTS OF THE ENDOCANNABINOIDS SYSTEM

The term endocannabinoid system refers to a sophisticated cellular signalling network, whose study began at the end of the 20th century (**Box 3**), comprising three principal components: i) cannabinoid receptors (CBRs); ii) their endogenous ligands (endocannabinoids); and iii) the enzymatic machinery governing their synthesis, degradation and transport (**Figure I6**).

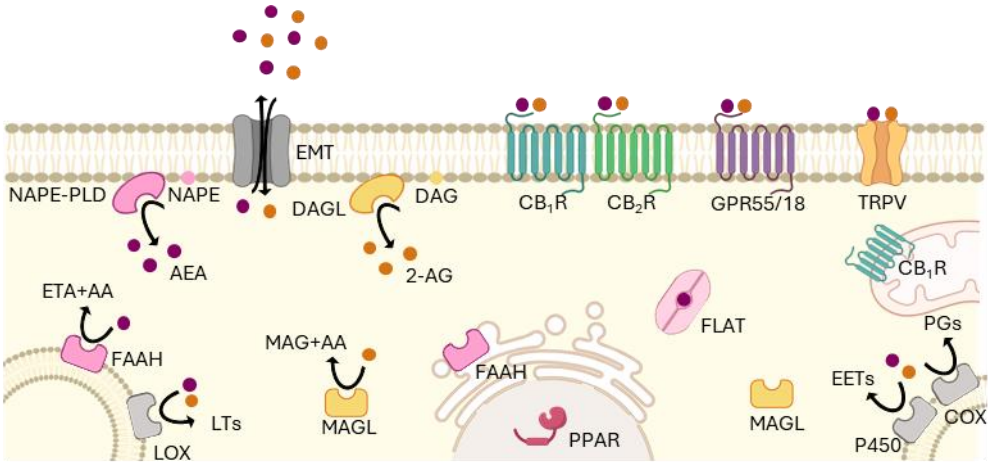

Figure I6. Components of the endocannabinoid system. Endocannabinoids [anandamide (AEA, N-arachidonoylethanolamine) and 2-arachidonoylglycerol (2-AG)] are synthetised from membrane phospholipids, with their metabolic machinery primarily comprising membrane-associated enzymes [biosynthetic enzymes: N-arachidonoylphosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL); hydrolytic enzymes: fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL); and oxidative enzymes: cyclooxygenase (COX), lysyl oxidase (LOX) and cytochrome P450 (P450)]. Their bioavailability may be modulated by binding to fatty-acid binding proteins, such as the FAAH-like anandamide transporter (FLAT), while their release is thought to be mediated by an unidentified endocannabinoid membrane transporter (EMT). Canonical [cannabinoid receptor 1 (CB_1R) and 2 (CB_2R)] and non-canonical receptors [GPR55, GPR18 and transient receptor potential vanilloid (TRPV) channel] are predominantly localised to cellular membranes, except for the transcription factor peroxisome-proliferator-activated receptor (PPAR), which resides in the nucleus. AA: arachidonic acid; EETs: epoxyeicosatrienoic acids; ETA: ethanolamine; LTs: leukotrienes; MAG: monoacylglycerol; PGs: prostaglandins.

Box 3. Cannabis and the endocannabinoid system

Cannabinoids, hydrophobic phytochemicals derived from the plant *Cannabis sativa* L. (commonly referred to as marijuana), have been used for millennia in religious, recreational and medicinal purpouses. Research into the underlaying chemistry of cannabis began in the mid-nineteenth century, prompted by observations of its behavioural effects across several animal species, including humans (Mechoulam et al., 2014). However, due to technical limitations, the first successful isolation of a pure active compound, cannabinol, was not achieved until the late 19th century, and its structural elucidation was delayed until the 1930s (R. Adams, 1942; Todd, 1946). A transformative breakthrough occurred in the 1960s with the advent of advanced isolation and analytical methodologies, enabling the structural characterisation of the two principal phytocannabinoids by Mechoulam and colleagues: cannabidiol (CBD), the major non-psychoactive constituent and exhibiting low cannabinoid receptor affinity (see below) (Mechoulam & Shvo, 1963), and Δ^9-tetrahydrocannabinol (THC), a partial agonist of both receptors (see below) and the main responsible of cannabis psychoactive effects (Gaoni & Mechoulam, 1964). In addition to these three cannabinoids, the plant produces more than 150 additional cannabinoid molecules, collectively accounting for over 20 % of cannabis-derived phytochemicals. Among these minor phytocannabinoids are cannabigerol, cannabichromene and tetrahydrocannabivarin, just to name a few (Schurman et al., 2019).

Mechanistic insights emerged decades later with the discovery that THC inhibits adenylate cyclase activity, implicating G-protein coupled receptor (GPCR) mediation (Howlett & Fleming, 1984). Subsequent studies confirmed the existence of a selective cannabinoid receptor [cannabinoid receptor type 1 (CB_1R)] in rat brain membranes (Devane et al., 1988), culminating in its molecular cloning in 1990 (Matsuda et al., 1990). Three years later, a second receptor was characterised, thus named cannabinoid receptor type 2 (CB_2R) (Munro et al., 1993). Concomitantly, investigations into endogenous receptor ligands (endocannabinoids) were initiated, hypothesizing lipid-like structure analogous to THC. This rationale led to the identification of the first endocannabinoid, N-arachidonoylethanolamide (anandamide/AEA), by Dr. Mechoulam's research group (Devane et al., 1992), which then isolated a second arachidonate-derived endocannabinoid, 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995). Systemic conceptualization of these findings led to the formal designation of the endocannabinoid system and subsequent research have established its involvement in diverse physiological processes including energy metabolism, nociception, memory, motor behaviour and immunity (Pacher et al., 2006).

CANNABINOID RECEPTORS

Considered the sole canonical CBRs, CB_1R and CB_2R demonstrate remarkable evolutionary conservation, being expressed in all vertebrates and select invertebrates, suggesting a critical role in homeostasis. Belonging to the class A GPCR superfamily, they exhibit moderate sequence homology (44 % overall identity, rising to 68 % within transmembrane domains) (Munro et al., 1993). Key structural divergences occur in the N-terminal extracellular loop II (critical for ligand binding) (Shao et al., 2016) and the intracellular C-terminal domain (Montero et al., 2005). Further characterisation has been achieved with the determination of the ligand-bound crystal structures of both CB_1R (Hua et al., 2016; Shao et al., 2016) and CB_2R (X. Li et al., 2019; Xing et al., 2020), providing unprecedented insights into their activation mechanisms (**Figure I6**).

The human *Cnr1* gene (encoding CB_1R) is located on chromosome 6 and comprises four exons, with only exon 4 encoding a 472-amino acid polypeptide (53 kDa) that undergoes post-translational glycosylation to yield a 64 kDa mature CB_1R (C. Song & Howlett, 1995). Initially described as the "central" CBR due to is abundance in the central nervous system—explaining the role of the ECS in the regulation of cognition, memory, and nociception—subsequent studies have revealed a broader tissue distribution (cardiovascular and

reproductive systems, liver, muscles, etc.), reflecting pleiotropic physiological roles (Maccarrone et al., 2015).

The human *Cnr2* gene (encoding CB_2R) is located on chromosome 1 and contains three exons, but only exon 3 encodes a 360-amino acid protein. Dual promoter regulation in human and mouse orthologues drives tissue-specific isoforms (Q. -R. Liu et al., 2009). While traditionally designated as the "peripheral" CBR due to its abundance in cells and tissues of the immune system (Galiègue et al., 1995)—consistent with ECS-mediated immunomodulation—CB_2R exhibits functionally significant expression in the gastrointestinal tract, musculoskeletal and reproductive systems and distinct brain regions. Notably, CB_2R expression is frequently upregulated in pathological conditions, positioning the receptor as a promising therapeutic target (Rakotoarivelo et al., 2024).

CBRs are predominantly Gαi/o-coupled and ligand binding uncouples the heterotrimeric G proteins leading to adenylate cyclase inhibition and a subsequent reduction in intracellular cAMP levels (Felder et al., 1995), as well as to the modulation of several ion channels conductance (inhibition of voltage-gated calcium channels and activation of G protein-gated inwardly rectifying potassium channels) (Mackie et al., 1995). Regulation of CBR's activity is mediated by its phosphorylation by G protein-coupled receptor kinases followed by binding of β-arrestins. This not only drives receptor desensitisation and internalisation but also establishes scaffolding platforms for non-G protein-mediated signalling pathways (Ibsen et al., 2017). Nonetheless, CBR signalling is considerably more complex than initially appreciated. For example, profound context-dependence and ligand-specific biased agonism has been described, whereby different ligands acting on the same receptor within the same cell type can elicit different cellular responses (Ibsen et al., 2017). Moreover, the ability of CBRs to form homo- and heterodimers with other receptors, including the tyrosine kinase receptor HER2 (Blasco-Benito et al., 2019; Pérez-Gómez et al., 2015), further diversifies the spectrum of intracellular responses triggered by a ligand.

Beyond the two well-established CBRs, cannabinoids and related molecules exhibit activity, albeit at higher concentrations, at additional molecular targets, including GPR55, GPR18, transient receptor potential vanilloid 1 (TRPV1) channel, and peroxisome-proliferator-activated receptor α (PPARα) (**Figure I6**). However, these targets fail to meet the strict criteria established by the IUPHAR for classification as canonical CBRs and further research is needed to elucidate their precise role in physiological processes and pathological mechanisms (Pacher et al., 2020).

ENDOCANNABINOIDS AND THEIR METABOLIC MACHINERY

The discovery of CBRs propelled research into their potential endogenous ligands (endocannabinoids, **Box 3**), with AEA and 2-AG emerging as the first and most extensively characterised agonists (**Figure I6**). These lipid-derived mediators, structurally derived from arachidonic acid (AA), act as partial (AEA) and full (2-AG) CB_1R and CB_2R agonists, respectively (Mechoulam et al., 2014). Based on the structural elucidation of AEA and 2-AG, subsequent investigations revealed additional endogenous lipids within the N-acylethanolamine and monoacylglycerol families that can act as CBRs, although these secondary endocannabinoids fall beyond the scope of this thesis (Rezende et al., 2023).

Functionally characterised as atypical retrograde neurotransmitters, endocannabinoids modulate synaptic transmission through postsynaptic-to-presynaptic signalling and are synthesised *de novo* upon demand rather than stored in vesicles (Rezende et al., 2023). Although four biosynthesis routes have been reported for AEA, it is primarily generated via transfer of an acyl group by *N*-acyl-transferase to AA-containing phosphatidylethanolamines, producing N-arachidonoylphosphatidylethanolamine (NAPE). This is followed by hydrolysis of NAPE by NAPE-specific phospholipase D (NAPE-PLD) to generate AEA. In contrast, 2-AG synthesis involves phospholipase C-mediated cleavage of glycerophospholipids to yield 1,2-diacylglycerol (DAG), which is subsequently converted into 2-AG by DAG lipase (DAGL) (Simard et al., 2022).

Following biosynthesis, endocannabinoids are immediately released, potentially via an unidentified membrane transporter. Their signalling is transient due to rapid cellular reuptake followed by enzymatic degradation: fatty acid amide hydrolase (FAAH) hydrolyses AEA to AA and ethanolamine, whereas monoacylglycerol lipase (MAGL) catabolises 2-AG to AA and glycerol. As eicosanoid precursors, endocannabinoids can also undergo oxidative metabolism via COX2, LOX, and cytochrome P450 enzymes, thereby generating bioactive metabolites that extend their physiological roles beyond classical receptor activation (Simard et al., 2022).

Recent investigations have revealed additional regulatory mechanisms governing endocannabinoids bioavailability, as their hydrophobic nature does not allow them to freely diffuse in the cytosol. This process may involve multiple molecular mediators, including heat shock proteins, cholesterol, ceramides, serum albumin, and other fatty-acid binding proteins such as the FAAH-like anandamide transporter (Moreno et al., 2019).

THE ENDOCANNABINOID SYSTEM AND CANCER

The ECS is critically involved in regulating numerous physiological processes, including reproductive, digestive, nervous, immune and metabolic functions among others. Consequently, its dysregulation has been implicated in a wide range of pathologies (e.g., neurodegenerative and autoimmune disorders, cancer, etc.), although causal relationships remain incompletely elucidated. This positions the ECS as a promising source of prognosis-predictive biomarkers, as well as potential therapeutic targets.

ALTERATIONS OF THE ENDOCANNABINOID SYSTEM IN CANCER

CBR expression is generally upregulated in tumour tissue compared to non-transformed tissue, correlating with higher disease grade and poorer patient prognosis. However, conflicting data report associations between reduced CBR expression levels and favourable clinicopathological features, suggesting that these alterations are context- and tumour-dependent (**Table I1**). Overexpression of CB_1R and/or CB_2R has been linked to adverse clinical outcomes in BC (Caffarel et al., 2006), glioblastoma (C. Sánchez et al., 2001), prostate cancer (Cipriano et al., 2013; Orellana-Serradell et al., 2015), colorectal cancer (Jung et al., 2013; Martínez-Martínez et al., 2015), pancreatic cancer (Carracedo et al., 2006; Michalski et al., 2008), and gynaecological malignancies (Messalli et al., 2014). Conversely, higher CBR expression has been associated to improved DFS rates in hepatocellular

carcinoma (X. Xu et al., 2006), BC (Elbaz et al., 2017) and tongue squamous cell carcinoma (Theocharis et al., 2016).

Specifically in BC, pioneering work by Caffarel and colleagues demonstrated reduced CB_1R mRNA levels alongside elevated CB_2R expression in tumour samples compared to non-malignant tissue, the latter correlating with higher histological tumour grades and markers of prognostic/predictive significance, notably HER2 overexpression (Caffarel et al., 2006, 2010). Subsequent studies by the same group elucidated CB_2R's pro-tumoural role through its physical interaction with HER2, forming a novel signalling platform that amplifies HER2-driven oncogenic signalling (Pérez-Gómez et al., 2015). This HER2-CB_2R heterodimer also represents a new therapeutic target, as its disruption triggers potent antitumoural responses (Blasco-Benito et al., 2019).

Other components of the ECS—both canonical and non-canonical—are also dysregulated in cancer (**Table I1**). Overexpression of the orphan receptor GPR55 is linked to enhanced disease aggressiveness and poor prognosis across multiple *in vitro* and *in vivo* models of glioblastoma, breast and pancreatic cancer (Andradas et al., 2011, 2016), ovarian and prostate cancer (Piñeiro et al., 2011), skin carcinoma (Pérez-Gómez et al., 2013), and colorectal cancer (Hasenoehrl et al., 2018). Conversely, TRPV2 expression is elevated in TNBC compared to normal tissue, correlating with improved prognosis and enhanced chemotherapy responsiveness (Elbaz et al., 2018), while exhibiting opposite prognostic implications in oesophageal cancer (Kudou et al., 2019).

Regarding endocannabinoids and their metabolic machinery (**Table I1**), the available data remain contradictory, and thus their precise role in cancer progression is not yet fully elucidated. In BC, evidence on endocannabinoids levels is scarce, with only one study reporting increased levels of NAPE, the precursor of AEA (P. C. Schmid et al., 2002). Concerning the metabolic enzymes, the limited literature focuses primarily on the degrading pathways: MAGL expression patterns appear inconsistent across studies (Gjerstorff et al., 2006; Simsek et al., 2023), whereas FAAH has been identified as a predictor of long-term survival in patients with luminal B BC (Tundidor et al., 2023).

Cancer	ECS component	Type of contribution/Implication	References
Glioblastoma	CB_1R, CB_2R	↑ expression in tumoural tissue ↑ CB_2R linked to tumour malignancy	(Sánchez et al., 2001)
	GPR55	↑ GPR55 linked to higher histological grade	(Andradas et al., 2011)
Prostate	CB_1R, CB_2R	↑ expression in advanced stages	(Cipriano et al., 2013; Orellana-Serradell et al., 2015)
	GPR55	GPR55 promotes cancer cell proliferation	(Piñeiro et al., 2011)
Colorectal	CB_1R	↑ CB_1R is a bad prognosis factor	(Jung et al., 2013)
	CB_2R	↑ CB_2R correlates with tumour growth	(Martínez-Martínez et al., 2015)
	GPR55	GPR55 promotes tumour growth	(Hasenoehrl et al., 2018)
Pancreatic	CB_1R, CB_2R	↑ expression in tumoural tissue	(Carracedo et al., 2006)
	CB_1R	↑ CB_1R linked to bad prognosis	(Michalski et al., 2008)
	GPR55	↑ GPR55 linked to more advanced stages	(Andradas et al., 2011)
Ovarian	CB_1R	↑ expression in tumoural tissue and linked to disease severity	(Messalli et al., 2014)
	GPR55	GPR55 promotes cancer cell proliferation	(Piñeiro et al., 2011)
HCC	CB_1R, CB_2R	↑ expression improves DFS	(X. Xu et al., 2006)
Tongue squamous cell carcinoma	CB_1R, CB_2R	↑ expression improves DFS and OS	(Theocharis et al., 2016)
Skin	GPR55	↑ expression in tumoural tissue and linked to tumour growth	(Pérez-Gómez et al., 2013)
Oesophageal	TRPV2	↑ expression linked to poor prognosis	(Kudou et al., 2019)
Breast	CB_1R, CB_2R	↓ CB_1R and ↑ CB_2R expression in tumoural tissue ↑ CB_2R correlates with disease severity CB_2R is overexpressed in HER2-enriched tumours	(Caffarel et al., 2006, 2010)
	CB_2R	CB_2R heteromerized with HER2 and contributes to its pro-oncogenic signalling	(Pérez-Gómez et al., 2015)
	CB_2R	CB_2R-HER2 disruption triggers antitumoural responses	(Blasco-Benito et al., 2019)
	CB_2R	↑ CB_2R linked to better recurrence free survival	(Elbaz et al., 2017)
	GPR55	↑ GPR55 linked to worse prognosis	(Andradas et al., 2011, 2016)
	TRPV2	↑ expression in tumoural tissue and linked to good prognosis and response to chemotherapy	(Elbaz et al., 2018)
	NAPE	↑ expression in tumoural tissue	(P. C. Schmid et al., 2002)
	MAGL	↑ MAGL expression in tumoural tissue and linked to metastasis	(Gjerstorff et al., 2006)
	MAGL	↓ MAGL expression in tumoural tissue and linked to metastasis	(Simsek et al., 2023)
	FAAH	FAAH is a predictor of long-term survival in luminal B BC	(Tundidor et al., 2023)

Table I1. Summarized review of the alterations of the ECS in several cancer types. ↑ and ↓ denote higher and lower expression, respectively. Expression in tumoural tissue is compared to that in non-transformed tissue o between different histological grades. HCC: hepatocellular carcinoma.

THERAPEUTICAL TARGETING OF THE ENDOCANNABINOID SYSTEM IN CANCER

Cannabinoids have well-established palliative benefits in oncology, including appetite stimulation, analgesia, and antiemesis—particularly in the management of chemotherapy-induced nausea and vomiting (CINV). In fact, in 1985 the FDA approved the use of nabilone and dronabinol, synthetic THC analogues, for the management of CINV in patients unresponsive to conventional antiemetics (Sexton et al., 2021). Current research focuses mainly on nabiximols, a THC:CBD 1:1 oromucosal spray with promising results in phase II trials for refractory CINV management (Grimison et al., 2020).

However, the therapeutic effects of cannabinoids on cancer patients may extend beyond symptom management. A substantial body of evidence indicates that activation of the ECS—whether via CBRs activation by endocannabinoids, phytocannabinoids or synthetic cannabinoids, or through inhibition of endocannabinoid's degradative enzymes—triggers potent antitumoural responses across a wide range of experimental cancer models, including cancer cell lines and genetically engineered mouse models (Hinz & Ramer, 2022). The first evidence of this potential dates back to 1975, when Munson and collaborators demonstrated that THC reduced the growth of Lewis lung adenocarcinoma cells both *in vitro* and *in vivo* (Munson et al., 1975). This and numerous subsequent studies have demonstrated that cannabinoid antitumoural action relies on i) the inhibition of cancer cell proliferation and induction of apoptosis, ii) the impairment of angiogenesis, and iii) the blockade of tumour invasion and metastasis (**Figure I7**).

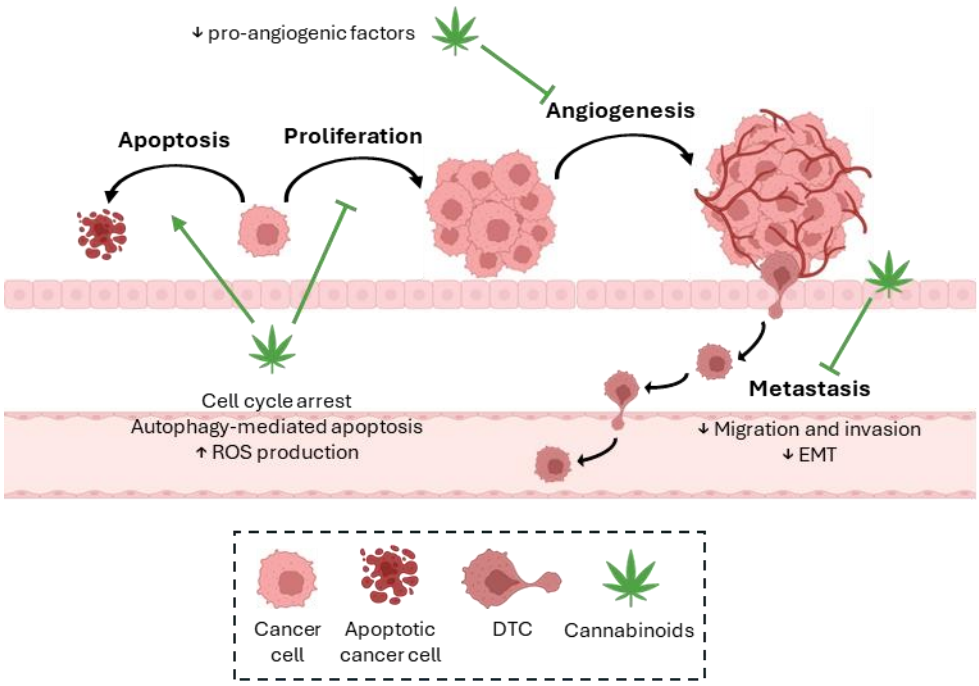

Figure I7. Antitumoural effects of cannabinoids. Cannabinoids inhibit tumour progression by impairing uncontrolled cancer cell growth—primarily through induction of cancer cell cycle arrest and promotion of apoptosis—as well as by suppressing angiogenesis and preventing metastasis. DTC: disseminated tumour cell; EMT: epithelial-to-mesenchymal transition; ROS: reactive oxygen species.

Blockade of cell proliferation and apoptosis induction

Cannabinoids, mainly through activation of CBRs, have been shown to reduce the proliferation of different cancer cells by induction of cell cycle arrest (Blázquez et al., 2006; Mimeault et al., 2003; J. M. Park et al., 2011; D. Xu et al., 2015). For example in BC, cell cycle progression has been shown to be inhibited by multiple mechanisms, such as downregulation of Cdc2 (major cyclin-dependent kinase controlling the entrance in mitosis) (Caffarel et al., 2006), inhibition of the pro-survival protein kinase B (PKB/Akt) (Caffarel et al., 2010), or disruption of S phase exit due to impaired Cdk2 activity (Laezza et al., 2006), among many others. Regarding to the induction of apoptosis, the most extensively studied tumour type is glioblastoma, and the mechanistic base involves the hydrolysis of sphingomyelin to ceramide (Galve-Roperh et al., 2000; C. Sánchez et al., 1998). The accumulation of this sphingolipid triggers an endoplasmic reticulum stress response, which entails upregulation of the transcriptional co-activator nuclear protein 1 and its effector, the pseudokinase tribbles homologue 3. This cascade culminates in the inhibition of the mammalian target of rapamycin complex 1 and the subsequent activation of autophagy-mediated cell death (Salazar et al., 2009). This effect appears to be common to other tumour types, such as pancreatic cancer (Carracedo et al., 2006) and hepatocellular carcinoma (Vara et al., 2011). Inhibition of tumour growth has also been validated in animal models of BC (Caffarel et al., 2010; Elbaz et al., 2015; Qamri et al., 2009) and hepatocellular carcinoma (Vara et al., 2011), among many others.

Additionally, CBR-independent mechanisms have been reported for the antiproliferative and proapoptotic effects of cannabinoids. For instance, CBD can induce apoptosis and autophagy in BC cells by enhancing the production of ROS (Shrivastava et al., 2011), while it inhibits glioblastoma cell proliferation via TRPV2 activation (Nabissi et al., 2013). Moreover, AEA may induce apoptosis of glioma cells through COX2 (Hinz et al., 2004) or TRPV1 (Contassot et al., 2004).

Although the vast majority of the studies show a robust antiproliferative effect of cannabinoids, some authors have proposed that there may be a bimodal concentration-dependent action of these molecules and low concentrations of cannabinoids may actually promote tumour cell proliferation both *in vitro* (Hart et al., 2004; Miyato et al., 2009; M. G. Sánchez et al., 2003) and *in vivo* (Martínez-Martínez et al., 2016).

Blockade of angiogenesis

Tumour neovascularization is essential for supplying oxygen and nutrients, as well as for facilitating waste removal in the growing tumour. Cannabinoid treatment has been shown to suppress this process by acting on both tumour and endothelial cells. On the one hand, cannabinoids reduce the expression of pro-angiogenic factors (e.g., VEGF) as well as their corresponding receptors (Blázquez et al., 2004; Casanova et al., 2003; Picardi et al., 2014; Portella et al., 2003), while increasing the expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (Ramer et al., 2014) in tumour cells. On the other hand, cannabinoids inhibit vascular endothelial cell migration and/or proliferation (Blázquez et al., 2003)

However, pro-angiogenic effects of cannabinoids have also been described. For instance, nanomolar concentrations of AEA stimulate endothelial cell proliferation via

CB_1R, which is overexpressed during angiogenesis (Pisanti et al., 2011). Similarly, in a colorectal cancer model, treatment with THC enhances the production of pro-angiogenic factors by tumour cells (Luo et al., 2022).

Blockade of cell invasion and metastasis

Metastasis constitutes the principal cause of cancer-related mortality, arising from the capacity of certain malignant cells to detach from the primary tumour mass, migrate and invade distant tissues. *In vitro*, activation of CBRs in cancer cells has demonstrated anti-invasive properties, either through upregulation of TIMP-1 or by downregulating MMP expression (Blázquez et al., 2008; Elbaz et al., 2015). In CBD-treated BC cells, suppression of the transcription factor Id-1 and its downstream target Sox-2 has been associated with antimetastatic effects (Soroceanu et al., 2013). Proteins governing cell adhesion and motility, particularly those involved in actin cytoskeleton reorganisation, are also modulated by cannabinoids: the activity of the small guanosine triphosphatase RhoA is impaired following activation of either CB_1R (Laezza et al., 2008) or CB_2R (Scarlett et al., 2018). EMT, a process intimately linked to metastasis, is influenced by AEA or its analogue methyl-F-AEA, which enhance epithelial marker expression (e.g., E-cadherin), while concomitantly reducing levels of mesenchymal markers (e.g., vimentin) (Laezza et al., 2012). *In vivo*, cannabinoids have been shown to diminish the formation of distant mass metastases in xenograft models of lung cancer (Portella et al., 2003), melanoma (Blázquez et al., 2006), and BC (Caffarel et al., 2010; Grimaldi et al., 2006).

Interestingly, one study has reported a cell-specific effect of cannabinoids on the invasive capacity of glioblastoma cells: the selective CB_2R agonist JWH-133 increased the invasion in the U138 cell line, reduced it in LN229, and had no impact on U87 cells (Hohmann et al., 2017).

Of interest, CB_2R seems to be the principal mediator of the antitumoural activity of cannabinoids in BC as its activation has been shown to induce cancer cell cycle arrest (Caffarel et al., 2006) and to reduce both tumour growth and metastasis spread in a genetically engineered mouse model of metastatic HER2-enriched BC (Caffarel et al., 2010), among many other effects.

Importantly, cannabinoids exhibit selective cytotoxicity towards tumour cells and may therefore complement or enhance the effect of current standard anti-neoplastic therapies. Preclinical evidence indicates that both THC and CBD can act synergistically with chemotherapeutic agents *in vitro*, as demonstrated in studies using bladder (Whynot et al., 2023) and leukaemia (Scott et al., 2017) cell lines, for example, and *in vivo*, notably in xenograft models of head and neck squamous cell carcinoma (Go et al., 2020) and glioblastoma (López-Valero et al., 2018; Torres et al., 2011), as well as in combination with radiotherapy in murine glioma models (Scott et al., 2014). Furthermore, some studies have shown that botanical cannabis extracts, as opposed to pure cannabinoids, may exert more potent antitumour effects due to the synergism among various plant constituents. For example, CBD-rich cannabis extracts showed more potency than CBD and synergism with chemotherapeutic agents in reducing the growth of human prostate cancer xenografts (De Petrocellis et al., 2013), and a THC-rich extract proved to be more potent than THC and

showed no interference with standard treatments in animal models of all the BC subtypes (Blasco-Benito et al., 2018). Conversely, and although CBD alone triggers antitumour responses, this cannabinoid has been reported to antagonise the effects of platinum-based chemotherapeutic agents in selected glioblastoma cell lines (Deng et al., 2017), and bladder cancer (Whynot et al., 2023) and melanoma cell models (Marzęda et al., 2022). These contradictory outcomes may be attributed to differences in experimental parameters such as the concentration of cannabinoid employed [with lower concentrations more likely to interfere with other treatments (Whynot et al., 2023)] and the timing of cannabinoid administration relative to chemotherapy (before, during, or after treatment). In *in vivo* studies, it is also important to consider cannabinoid metabolism. Phase I metabolism of cannabinoids is primarily catalysed by the cytochrome P450 complex, particularly CYP3A4, which is also responsible for the metabolism of more than 50 % of medicines. Indeed, a potential interaction between CBD and tamoxifen has been reported, resulting in in decreased levels of the active metabolite of the latter, although no clinical effect has yet been demonstrated (Buijs et al., 2023; Parihar et al., 2022).

Clinical trials

The robust preclinical evidence of the antitumoural effects of cannabinoids provided the foundation for a pilot clinical trial involving nine patients with recurrent glioblastoma. In this study, the intracranial administration of THC was found to be safe and well tolerated, although it produced only a modest improvement in OS. However, due to the study's design limitations and small sample size, these results should be interpreted with caution (Guzmán et al., 2006).

In 2021, Twelves and colleagues reported the results of a phase Ib trial in which the secondary objective was to assess the antitumour efficacy of a cannabinoid-based drug (nabiximols) in combination with temozolomide, the standard treatment for glioblastoma. Although the number of participants was limited (n = 21), the study proved safety and tolerability of nabiximols, and signs of efficacy in terms of increase OS at 1 and 2 years (Twelves et al., 2021). Building on these promising results, the phase II ARISTOCRAT trial (CrinicalTrials.Gov: NCT05629702, currently recruiting) has been launched to evaluate efficacy and safety of nabiximols plus temozolomide in a larger cohort of patients with recurrent glioblastoma (Bhaskaran et al., 2024).

Also in 2021, Schloss and co-workers published the results of a randomised phase II clinical trial comparing two different ratios of medicinal cannabis in patients with high-grade gliomas (n = 88). While no placebo group was included, a retrospective control group was used for comparation. The study provided preliminary evidence of antitumour activity: tumour size was reduced in 11 % of patients in the cannabinoid-treated group vs 0 % in the control group and disease progression was observed in 27.5 % vs 53 % patients, respectively (Schloss et al., 2021).

In 2023, the Spanish Neuro-Oncology Group (GEINO) initiated a multicentre phase Ib clinical trial to evaluate the safety of combining naboximols, temozolomide and radiotherapy in patients with newly diagnosed glioblastoma (Clinicaltrials.gov: NCT03529448). Patient recruitment (n = 33) has been completed, and results are expected in December 2025.

THE ENDOCANNABINOID SYSTEM IN IMMUNE CELLS

The expression of CBRs by immune cells, together with their ability to synthesise, uptake and degrade endocannabinoids, underpins the role of the ECS—particularly CB_2R (Buckley et al., 2000)—as a key modulator of immune responses (Chiurchiù et al., 2015). CB_2R has been shown to exert a negative regulatory effect on inflammation, as evidenced, for example, by numerous studies in which CB_2R-deficient animals display a pro-inflammatory phenotype (reviewed in Rakotoarivelo et al., 2024).

A substantial body of *in vitro* and *ex vivo* evidence supports the immunosuppressive functions of cannabinoids (**Figure I8**), including reduced immune cell proliferation and/or induction of apoptosis, inhibition of migration, decreased production of pro-inflammatory cytokines, differentiation of immunosuppressive cells, etc. For example, THC suppressed T cell proliferation and shifted the cytokine prolife towards Th2 by a CB_2R-dependent mechanism (M. Yuan et al., 2002), while also inducing apoptosis in DCs via CB_1R and CB_2R (Do et al., 2004). This phytocannabinoid also reduced the killing capacity of macrophages—a phenomenon also observed with AEA (Cabral et al., 1995)—as well as of NK cells (Klein et al., 1987; Massi et al., 2000; Specter et al., 1986), although the specific molecular target was not identified in these cases. CBD induced the differentiation of Tregs (Dhital et al., 2017) and decreased antibody production by B cells (Jan et al., 2007) via unknown mechanisms. The endocannabinoid AEA inhibited T cell migration (Joseph et al., 2004) and the production of pro-inflammatory cytokines by DCs (Chiurchiù et al., 2013) through CB_2R.

Conversely, only a limited number of pro-inflammatory effects, mainly attributed to 2-AG, have been reported, such as leukocyte recruitment (Gokoh et al., 2005; Maestroni, 2004), release of pro-inflammatory mediators (Stefano et al., 2000), enhanced phagocytosis (Gokoh et al., 2007; Shiratsuchi et al., 2008) and DC maturation (Qiu et al., 2019).

Notably, tissue concentrations of endocannabinoids are typically in the nanomolar range (Di Sabatino et al., 2011), suggesting that effects observed at high micromolar concentrations *in vitro* may lack physiological relevance *in vivo*. Overall, current evidence points to a homeostatic immunoregulatory role of endocannabinoids, rather than a strictly immunosuppressive or immunostimulatory function. While most of these actions are mediated via CB_2R, and to a lesser extent CB_1R, some effects are independent of classical CBRs. For example, CBD ameliorated experimental autoimmune hepatitis by inducing MDSC differentiation through activation of PPARγ (V. Hegde et al., 2010) or TRPV1 (V. L. Hegde et al., 2011) or by triggering T cell apoptosis via ROS generation. Similarly, 2-AG metabolite generated through COX enzymatic activity inhibited IL-2 secretion in activated T cells through PPARγ activation (Rockwell et al., 2006, 2008).

Inflammation is now recognised as a hallmark of cancer (Hanahan, 2022), therefore representing a therapeutic opportunity. In this context, cannabinoids have emerged as promising agents owing to their immunosuppressive properties. Indeed, their potential therapeutic benefit in chronic inflammatory diseases has been suggested, as evidenced, for example, by the amelioration of clinical signs in experimental autoimmune encephalomyelitis models through reduced microglial activation and T cell recruitment (Kozela et al., 2011). However, the intricate interplay between cannabinoids, inflammation and immune regulation in cancer underscores the need for further investigation before these compounds can be confidently translated into clinical oncology practice, ensuring both efficacy and safety for patients.

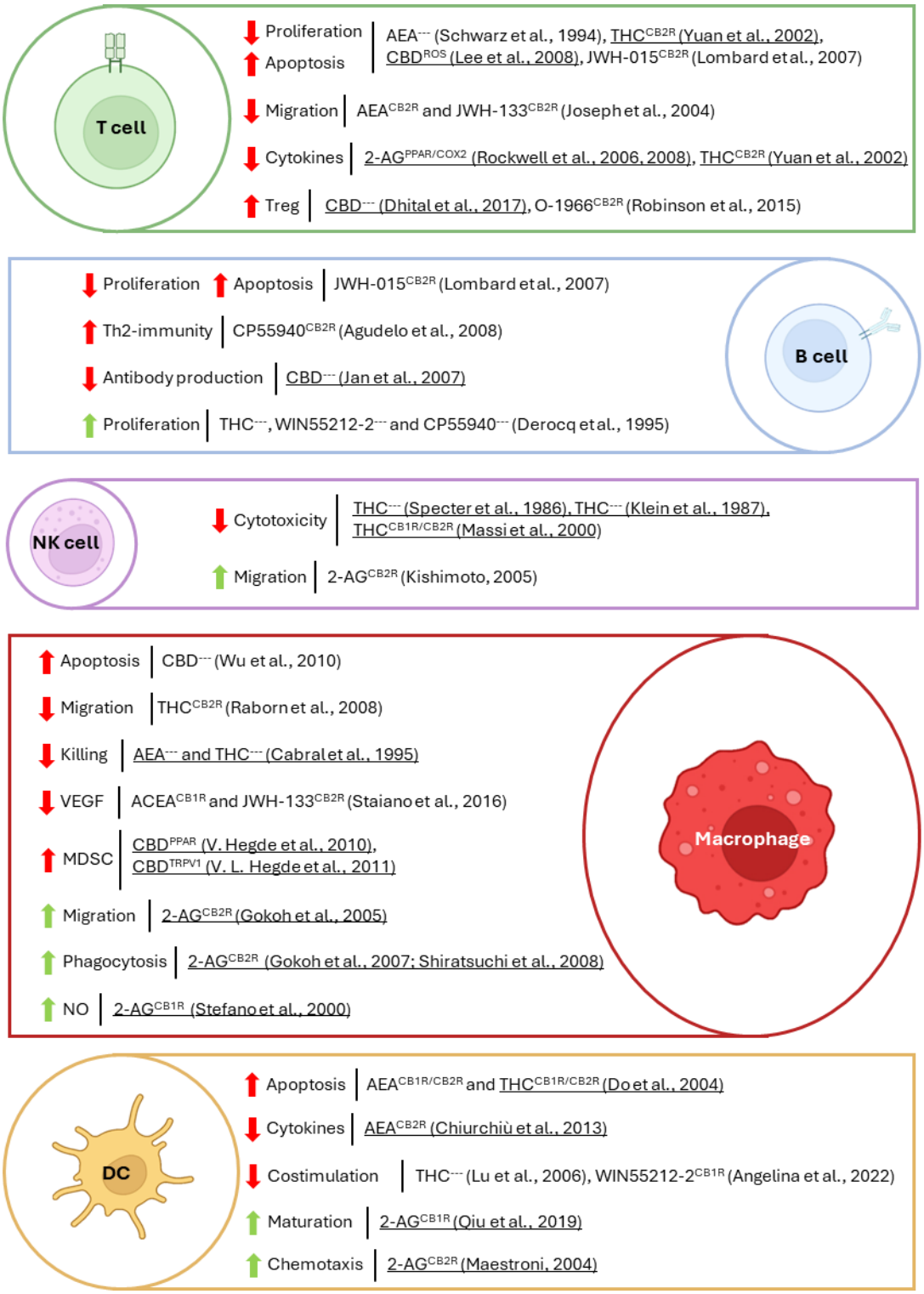

Figure I8. Summary of selected effects of cannabinoids on immune cells. Red arrows indicate immunosuppressive mechanisms, whereas green arrows denote pro-inflammatory effects. Superscripts indicate the proposed molecular targets; "---" denotes unknown targets. Underlined references correspond to those explicitly cited in the main text.

THE ENDOCANNABINOID SYSTEM IN THE TUMOUR IMMUNE MICROENVIRONMENT

In most of the studies discussed in the section "The endocannabinoid system and cancer", the expression of the ECS components was analysed in bulk tumour tissue (i.e., lacking cell-type-specific resolution) and the antitumoural activity of cannabinoids has been mainly evaluated in cancer cell cultures and/or animal models lacking a fully functional immune system. Very few investigations have been carried out in more robust cancer models, recapitulating the complexity of the tumour immune microenvironment (TIME). Thus, the role of the ECS expressed by the TIME in BC progression as well as the contribution of the TIME to the antitumour effects of cannabinoids remain unaddressed.

Regarding the physiological role of the ECS in the TIME (**Figure I9**), the use of genetically engineered mouse models lacking different elements of the ECS is an essential experimental approach. In a syngeneic melanoma model, deficiency of CB_2R within the TIME led to enhanced melanoma growth, which was associated with a notable infiltration of immature B cells and a significant reduction in CD8 T cell frequencies (Gruber et al., 2021). Similarly, in hepatocellular carcinoma CB_2R-deficient mice developed larger tumours compared to WT counterparts, a phenomenon potentially attributable to impaired immunosurveillance, as evidenced by reduced CD4 T cell infiltration (Suk et al., 2016). In carcinogen-induced colon cancer, CB_2R deficiency resulted in an increased number of dysplastic polyps, accompanied by elevated levels of MDSCs and diminished CD8 T cell infiltration (Iden, Raphael-Mizrahi, Awida, et al., 2023). Conversely, CB_2R genetic deletion has also been reported to slow down tumour growth in murine models of melanoma, colon cancer and Lewis lung adenocarcinoma, an effect that was paralleled by increased frequencies of CD8 T cells within the tumour (X. Xiong et al., 2022). This observation has been corroborated in non-small cell lung cancer (NSCLC), where genetic ablation of CB_2R led to reduced tumour burden and was associated with greater accumulation and cytotoxic activity of CD8 T and NK cells (Sarsembayeva et al., 2023). In colon cancer, deficiency of MAGL, acting through 2-AG–CB_2R signalling, promoted polarisation of macrophages towards M2-TAMs, which contributed to suppression of cancer-related CD8 T cells and enhanced tumour growth (W. Xiang et al., 2018). In the same tumour type, GPR55 knockout mice displayed lower MDSC infiltration, but a higher number of CD4 and CD8 T cells, thus having better prognosis (Hasenoehrl et al., 2018).

With respect to pharmacological modulation of the ECS in the TIME (**Figure I9**), some studies suggest that CB_2R activation may enhance tumourigenesis by interfering with immune surveillance. For example, THC exposure in murine models of breast (McKallip et al., 2005) and lung cancer (Zhu et al., 2000) has been shown to suppress cell-mediated Th1 responses and promote Th2-associated cytokines, or to impair the number and function of CD8 T cells in melanoma (X. Xiong et al., 2022). By contrast, in melanoma cells, the antitumour activity of CB_2R agonists appears to be independent of the immune status of the host (Blázquez et al., 2006). In murine TNBC, CBD treatment reduced recruitment of M2-TAMs both in primary tumours and metastases (Elbaz et al., 2015). Similar findings have been reported in melanoma, where THC antagonised the infiltration of pro-tumourigenic myeloid cells within the TIME (Glodde et al., 2015). In NSCLC, administration of the CB_2R agonist JWH-015 markedly reduced macrophage recruitment to the tumour, contributing to inhibition of tumour progression (Ravi et al., 2016). In pancreatic ductal adenocarcinoma, 2-AG administration promoted DC maturation and pro-inflammatory cytokine production

but also fostered an immunosuppressive TIME via increased MDSC accumulation (Qiu et al., 2019). The variability of these effects may be related to differential expression of CBRs: while tumours with high levels of these proteins are effectively killed by cannabinoids, in tumours with low or null expression of CB_1R or CB_2R—such as those reported in studies describing pro-tumoural effects, a scenario not typically observed in patients where CB_2R is usually upregulated—may experience enhanced growth due to suppression of the antitumour immune response.

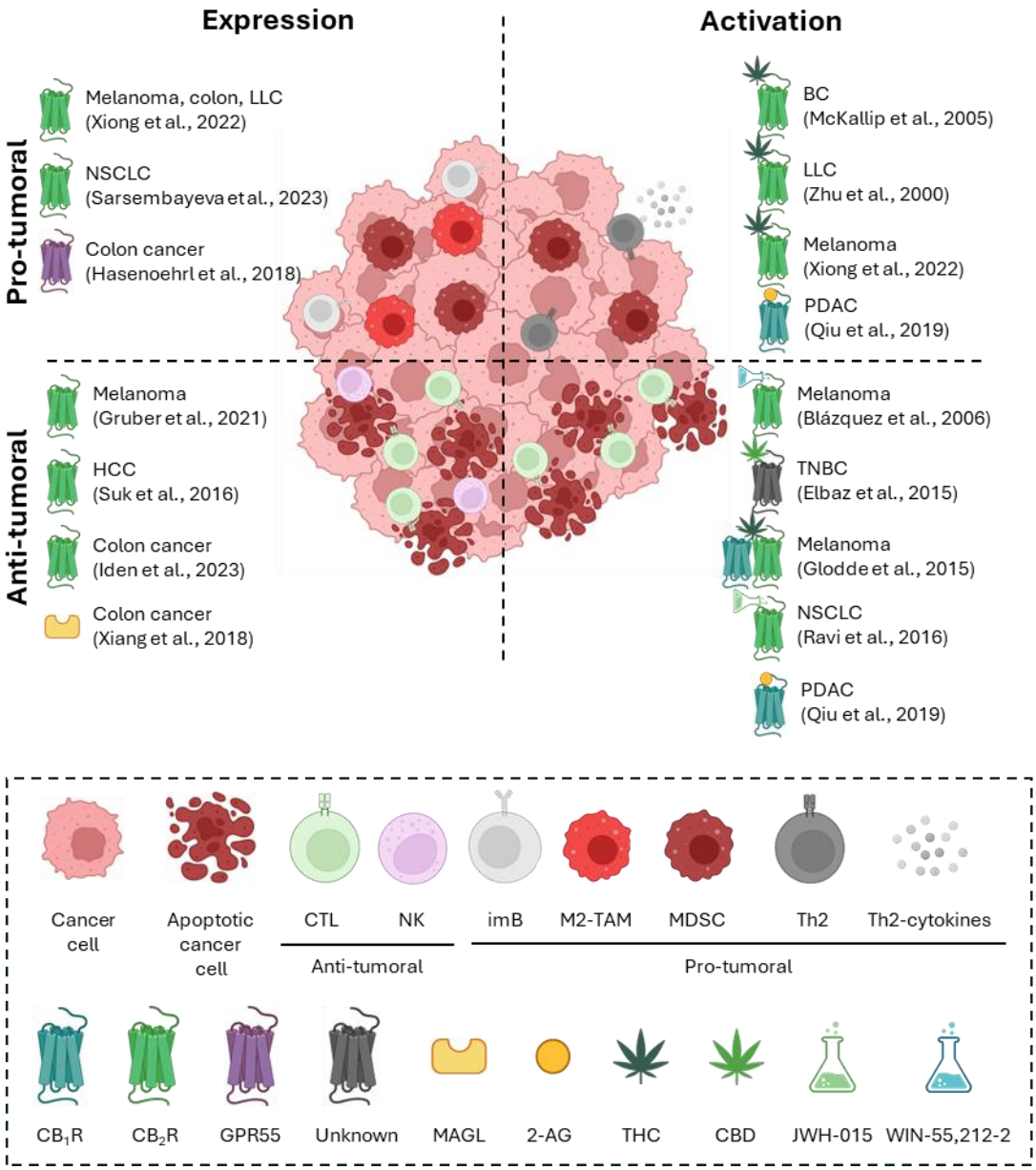

Figure I9. Summary of the main findings on the effect of the expression and/or activation of the ECS in the immune tumour microenvironment. The employed cannabinoid ligand and its molecular target are specified. LLC: Lewis lung adenocarcinoma; NSCLC: non-small cell lung cancer; HCC: hepatocellular carcinoma; PDAC: pancreatic ductal adenocarcinoma; imB: immature B cell.

As discussed previously in the context of BC, immunotherapy is emerging as a promising therapeutic strategy, particularly for TNBC patients. However, only a limited number of experimental and clinical studies have addressed the impact of cannabis on immunotherapy, a matter of potential concern given that cannabinoids are well-known immunosuppressive agents, whereas immunotherapy aims to reinvigorate antitumour immunity. Preclinical studies have shown that THC can reduce the efficacy of anti-PD-1 therapy (X. Xiong et al., 2022), whereas administration of a cannabis extract did not have a detrimental effect on the activity of anti-PD-L1 antibody (Waissengrin et al., 2023). Furthermore, CB_2R-deficient mice exhibit significantly improved responses to anti-PD-1 compared to WT animals (Sarsembayeva et al., 2023). Clinically, two observational studies have highlighted the immunosuppressive potential of cannabinoids as a concern in patients undergoing immunotherapy: concomitant use of ICI and cannabis was associated with reduced response rates in a first retrospective study (Taha et al., 2019) and shorter time to tumour progression and OS in a second prospective study (Bar-Sela et al., 2020). However, a later study that re-examined the reported data identified substantial inaccuracies that could affect the conclusions of those two studies (Piper et al., 2024); therefore, their findings should be interpreted with caution. More recently, a clinical study found that the concomitant use of medical cannabis with first line pembrolizumab monotherapy in patients with NSCLC did not negatively impact time to tumour progression. However, and although a *p* value of 0.08 was obtained for the statistical comparison of the two analysed groups, strictly meaning no differences between the two, the OS was nearly doubled in the cannabis-naive group (54.9 versus 23.6 months; 95 % confidence interval 0.99 to 2.51) (Waissengrin et al., 2023), pointing once again to extreme caution when interpreting these results and to the need of additional studies. Moreover, these studies included very heterogeneous groups of patients with various tumour types, the majority of whom had already advanced metastatic disease. Furthermore, a wide range of cannabinoid products was used, often with poorly defined dosages.

In summary, the current evidence does not allow for a definitive assertion of a deleterious effect of cannabinoids on ICI-based treatment outcomes. To elucidate the potential interaction between cannabis and ICIs, in-depth preclinical studies as well as randomised placebo-controlled trials with adequate statistical power and standardised cannabinoid preparations are required.

OBJECTIVES

HYPOTHESIS AND OBJECTIVES

The tumour microenvironment is a complex entity where cancer cells engage in complex and dynamic interactions with both cellular and non-cellular components to receive cues on how to survive, proliferate and spread to other organs. Among the cellular constituents, immune cells act as key players in the regulation of cancer progression, as they can exert either pro- or anti-tumourigenic effects depending on the context. These cells are known to express CB_2R, whose expression and activation have been shown to exert immunomodulatory, mainly immunosuppressive, effects in different scenarios. In addition, activation of this receptor in BC cells has been demonstrated to trigger potent antitumoural responses. Despite this well-established understanding of the function of CB_2R in the physiopathology of immune and cancer cells, its specific role within the tumour immune microenvironment remains poorly understood.

In this scenario, the **hypothesis** of this doctoral thesis is that CB_2R expressed by the TIME may impair antitumour immunity, thereby promoting BC progression, whereas its general pharmacological activation in tumours (i.e., in the TME and cancer cells simultaneously) may shift the balance toward antitumour responses.

To validate this hypothesis, we defined the following aims:

1. **Aim 1. Characterisation of CB_2R expression in immune cells.**
   This aim comprises two partial objectives:
   i) *In-silico* analysis of CB_2R expression in non-transformed tissues and immune cells.
   ii) *On vivo* analysis of CB_2R expression in immune cells, including those residing within the BTME.

2. **Aim 2. Analysis of the role of BTME-CB_2R in the initiation and progression of BC.**
   This aim consists of three partial objectives:
   i) Analysis of the role of TME-CB_2R in defining the baseline composition of splenic immune cells.
   ii) Assessment of the role of TME-CB_2R in the initiation and progression of BC.
   iii) Evaluation of the role of TME-CB_2R in the generation of lung metastases.

3. **Aim 3. Analysis of the simultaneous targeting of CB_2R in immune and cancer cells.**
   This aim encompasses two partial objectives:
   i) Assessment of the impact of CB_2R activation on immune cell functions.
   ii) Analysis of the simultaneous activation of CB_2R in both immune and BC cells.

MATERIALS AND METHODS

ANALYSIS OF PUBLIC DATASETS

CB_2R mRNA expression in human and mouse samples (tissues and isolated immune cells) was analysed in publicly available databases referenced throughout the results section.

Data from the Human Protein Atlas (HPA) project (Uhlén et al., 2015; v24.proteinatlas.org) regarding tissue expression of CB_2R mRNA were obtained from https://www.proteinatlas.org/humanproteome/tissue/data#hpa_tissues_rna; whereas data corresponding to isolated immune cells were retrieved from https://www.proteinatlas.org/humanproteome/single+cell/immune+cell/data#immune_cell. Data from the Genotype-Tissue Expression (GTEx) project (Lonsdale et al., 2013) were accessed through the Application Programming Interface GTEx API V2. Mouse immune cell data from the Immunological Genome Project (ImmGen) (Heng et al., 2008) and the Database of Immune Cell Expression project (DICE) (Schmiedel et al., 2018) were obtained from their official website: https://rstats.immgen.org/DataPage/ and https://dice-database.org/downloads, respectively.

To normalise CB_2R mRNA levels in tissues and isolated immune cells, the formula $\log_{10}(TPM+1)$ was applied, where TPM referred to transcripts per million. To assess differences in gene expression across study groups, the formula $\log_2(FC)$ was used, with FC representing the fold change.

CELL LINES AND CULTURE REAGENTS

All cell lines were routinely tested for *Mycoplasma* spp. contamination and maintained in a humidified atmosphere of 5 % CO_2 at 37 ºC. **Table MM1** lists the main characteristics of the cell lines used in this thesis.

Cell media were purchased from Sigma-Aldrich (St. Louis, Missouri, US); fetal bovine serum (FBS) and β-mercaptoethanol (βME, #21985-023) were from Gibco (Amarillo, Texas, US); L-Glutamine (L-Gln, #SH30034.01) was from Lonza (Basel, Switzerland); and Penicillin/Streptomycin (P/S, #30-001-CI) and Trypsin-EDTA 1X (0.25 % Trypsin/2.21 mM EDTA, #25053CI) were from Corning (Corning, New York, US).

Concanavalin A from *Conavalia ensiformis* (ConA) (Sigma-Aldrich, #C5275) was dissolved in phosphate saline buffer (PBS). Recombinant mouse interleukin 2 (rmIL-2) [PeproTech, Cranbury, New Jersey, US, #212-12B)] was dissolved in PBS containing 0.1 % bovine serum albumin (BSA) [NZYtech, Lisbon, Portugal, #MB47002]. The phytocannabinoid Δ^9-tretrahydrocannabinol (THC) (THC Pharm GmbH, Frankfurt, Germany, #THC1099) and the CB_2R-selective agonist HU-308 (Tocris Bioscience, Abingdon, United Kingdom, #3088) were dissolved in dimethylsulfoxide (DMSO) (PanReac Applichem, Darmstadt, Germany, #A3672) and, due to their lipophilic nature, stored in Eppendorf tubes precoated with Sigmacote (Sigma Aldrich, #SL2).

Cell line	Cellular type	Growth medium	Source of origin
EO771	Mouse breast carcinoma (Luminal B)	RPMI	Dr. Hector Peinado (CNIO)
N202-1A	Mouse breast carcinoma (HER2-enriched)	DMEM	Dr. Vincenzo Bronte (IOV)
AT3	Mouse breast carcinoma (TNBC)	DMEM	ATCC
AT3-luc-mCherry	AT3 derived cell line	DMEM	Homemade
HEK-293T	Human Embryonic Kidney	DMEM + 2 mM L-Gln	ATCC

Table MM1. Cell lines and culture conditions. All culture media were supplemented with 10 % FBS + 1 % P/S. ATCC: American Type Culture Collection (Barcelona, Spain). DMEM: Dulbecco's Modified Eagle's Medium. RPMI: Roswell Park Memorial Institute. CNIO: Spanish National Cancer Research Center, Madrid, Spain. IOV: Istituto Oncologico Veneto, Padova, Italy.

GENERATION OF AT3 BC CELLS EXPRESSING LUCIFERASE

AT3 BC cells stably expressing firefly luciferase and the red fluorescent protein mCherry (AT3-luc-mCherry) were generated by lentiviral transfection. Briefly, HEK-293T cells were seeded in antibiotic-free medium at 80 % confluency (1 x 10^6 cells per 60 mm dish). and allowed to attach to the plastic surface for 24 h. On day 2, cells were transfected with 2.9 µg of total DNA at a ratio 1:2:3 (envelope, packaging and transfer plasmids, respectively) (**Table MM2**) mixed in Optimem™ I Reduced Serum Medium (Gibco, #31985047) and with 4 µg/mL polyethylenimine (PEI) (Polysciences, Warrington, Philadelphia, US, #23966-100). Next day, HEK-293T were changed to the fresh DMEM and AT3 BC cells were seeded at 40 % confluency (3 x 10^5 cells per 60 mm dish). On day 4, HEK-293T supernatant was collected, centrifuged at 1200 rpm for 5' at room temperature (RT), filtered through a 0.45 µm filter unit (Dutscher, Bernolsheim, France, #051734), supplemented with 8 µg/mL hexadimethrine bromide (polybrene) (Sigma-Aldrich, #H9268) and finally added to the AT3 culture. The following day, cells were changed to fresh growth medium and transfection efficiency was monitored by fluorescence microscopy using mCherry protein expression. Finally, mCherry-positive cells were sorted on a BD FACSAriaTM III system (Becton Dickinson, Franklin Lakes, New York, US) at the Flow Cytometry and Fluorescence Microscopy Unit (Complutense University of Madrid (UCM), Madrid, Spain)

Prior to any experiment, luciferase expression was checked by incubating cells with 150 µg/mL D-luciferin (Goldbio, St. Louis, Missouri, US, #115144-35-9) for 5-10' at 37 ºC and then measuring the bioluminescence in an IVIS Spectrum system (Perkin Elmer, Waltham, Massachusetts, US).

Any material ever exposed to lentiviruses was safely inactivated using Virkon™ (Day.Impex, Colchester, UK, #12358667) before being disposed of as biohazardous waste.

Plasmid	Name	Origin
Envelope	pMD2.G	Addgene #12259
Packaging	psPAX2	Addgene #12260
Transfer	CSCW2-Fluc-ImC	Dra. Gema Moreno Bueno (UAM)

Table MM2. Plasmids used for lentiviral transfection and their origin. Addgene, Watertown, Massachusetts, US. UAM: Autonomous University of Madrid, Madrid, Spain

ANIMALS

Animals were housed at the animal facility of the School of Biology (UCM) and were offered ad libitum access to water and food. All procedures were performed with the approval of the UCM Animal Experimentation Committee and Madrid Regional Government, according to the EU official regulations.

FVB/NJ MMTVneu $CB_2R^{+/+}$ and $CB_2R^{-/-}$ mice were previously generated as described in Pérez-Gómez et al., 2015. C57BL/6 $CB_2R^{-/-}$ animals were kindly donated by Dr. María Sagrario Gómez Ruiz (UCM). C57BL/6 CB_2R^{eGFP} (C57BL/6-Cnr2tm1.1Geno/J) reporter mice were kindly donated by Dr. Julián Romero (Francisco de Vitoria University, Madrid, Spain). C57BL/6 CD4Cre (B6.Cg-Tg(Cd4-Cre)1Cwi/BfluJ) transgenic animals were kindly donated by Dra. Danay Cibrián Vera (Instituto de Investigación Sanitaria Hospital Universitario de la Princesa, Madrid, Spain).

To establish the C57BL/6 CB_2R full knockout colony ($CB_2R^{-/-}$) in our animal facility, C57BL/6 $CB_2R^{-/-}$ mice were crossed with C57BL/6 $CB_2R^{+/+}$ animals. The hemizygous offspring were then crossed to produce $CB_2R^{+/+}$ and $CB_2R^{-/-}$ littermates.

For the specific deletion of CB_2R in T cells, the strain CD4Cre:CB_2R^{eGFP} (CD4-$CB_2R^{-/-}$) was generated by crossing CD4Cre mice, which contain CD4 enhancer, promoter and silencer sequences driving the expression of a Cre recombinase gene, with CB_2R^{eGFP} mice, which possess loxP sites flanking exon 3 of the *Cnr2* gene. The specific deletion of the CB_2R protein in T cells was confirmed by flow cytometry analysis, as demonstrated in **Figure MM1**.

Animals were routinely genotyped for CD4Cre and/or CB_2R alleles by specific polymerase chain reactions (PCR) using NZYTaq II 2X Green Master Mix (NZYtech, #MB35803). Primers and the genotyping protocol provided by Jackson Laboratory were used for the CD4 allele, while for the CB_2R allele three primers were designed to distinguish the wild-type (WT) (153 bp), floxed (244 bp) and knockout (KO) (286 bp) alleles (**Table MM3 and Figure MM2**). PCR conditions were as follows: initial denaturation at 95 °C for 5'; 30 cycles of denaturation at 95 °C for 40'', annealing at 62 °C for 30'', and elongation at 72 °C for 30''; and a final extension at 72 °C for 7'.

GENERATION OF MAMMARY TUMOURS IN IMMUNOCOMPETENT MICE

BC cells in exponential growth phase were trypsinised, collected in complete medium and counted using a Neubauer chamber. After centrifugation, cells were resuspended at the desired concentration (10 x 10^6 cells/mL for N202-1A cell line and 20 x 10^6 cells/mL for EO771 and AT3 cell lines) in 50 % PBS – 50 % GFR Matrigel (Corning, #354230). Fiftly µL of cell suspension were orthotopically implanted into the fourth mammary fat pad of immunocompetent 8–10-week-old female mice (N202-1A in FVB/NJ MMTVneu females and EO771 and AT3 in C57BL/6 females). Animals were routinely palpated and as soon as tumours were detected their growth was monitored using a digital calliper. Tumour volume was calculated as $(4\pi/3)$ x $(width/2)^2$ x $(length/2)$ and was used to set 3 different endpoints for animal sacrifice and sample collection: T1) 100 – 200 mm^3; T2) 400 – 600 mm^3; and T3: 800 – 1000 mm^3. Harvested tumours were divided into two pieces that were used for i) flow cytometry analysis [digested (see below) and cryopreserved in freezing medium (90 % FBS + 10 % DMSO) until analysis] and ii) protein extraction (snap frozen).

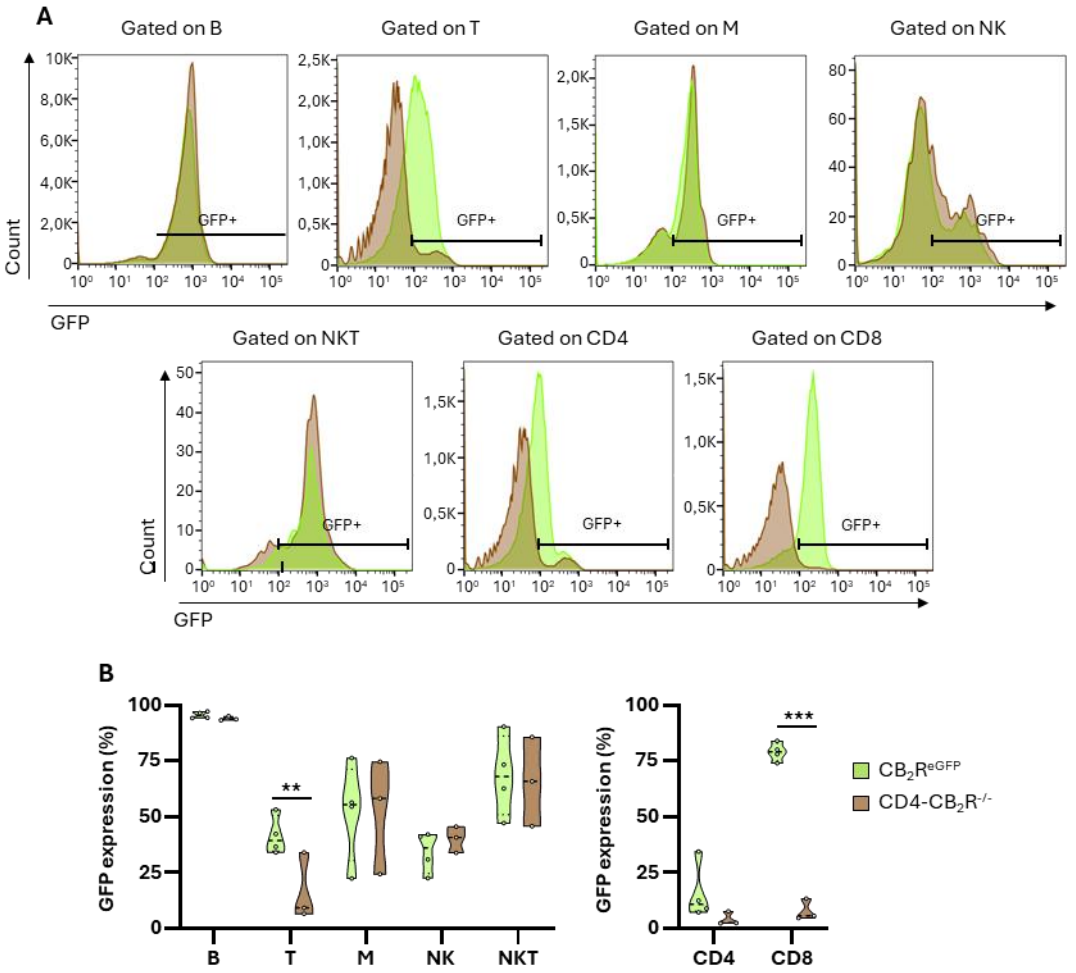

Figure MM1. Validation of the CD4-$CB_2R^{-/-}$ mouse model for CB_2R deletion in T cells. (A) Representative flow cytometry plots showing GFP (CB_2R) expression in distinct immune cell populations from CB_2R^{eGFP} and CD4-$CB_2R^{-/-}$ mice. (B) Quantification of GFP (CB_2R) expression from A. Splenocytes isolated from CB_2R^{eGFP} and CD4-$CB_2R^{-/-}$ females are shown in green and brown, respectively. M: myeloid cells; CD4: helper T cells; CD8: cytotoxic T cells. Student's t-test: ** $p < 0.01$, *** $p < 0.001$ vs CB_2R^{eGFP}.

Primer	Sequence (5' – 3')	Primer type
1	CCTGAAGTAAGAGGGTCTAACTG	KO forward
2	GCCAGGATCCAGAACTCCAG	WT/floxed forward
3	CCATTACCCTAGAACTGGCTTCA	Common reverse

Table MM3. Primers used for genotyping the CB_2R allele. Primers 1 and 3 allow amplification of the KO allele. Primers 2 and 3 allow amplification of the WT and floxed alleles, which differ in size by the insertion of two site-directed recombination sequences (**Figure MM2**).

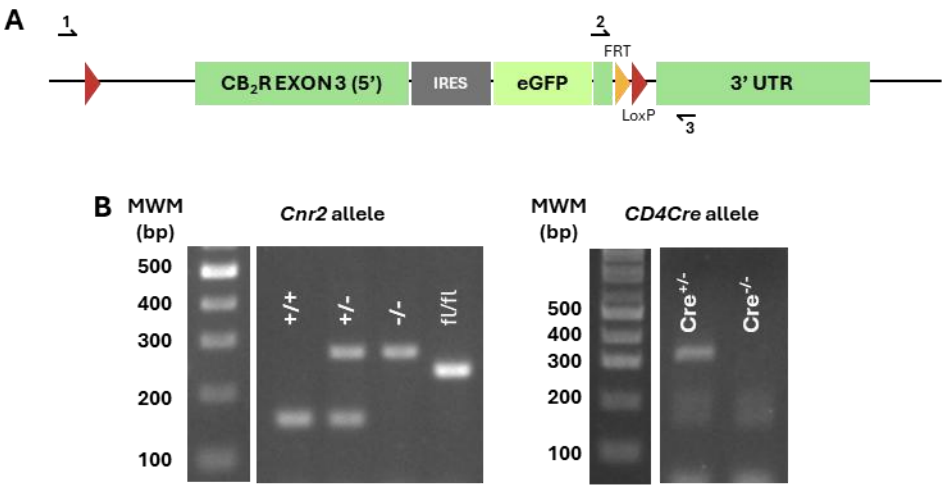

Figure MM2. Genotyping of $CB_2R^{-/-}$ and CD4-$CB_2R^{-/-}$ animals. (A) Primer design strategy for the identification of WT, floxed and KO alleles. (B) Representative images of the different genotype combinations for CB_2R (left) and CD4Cre (right) alleles. +/+: wild-type; +/-: heterozygous; -/-: knockout; fl/fl: floxed/floxed.

GENERATION OF LUNG METASTASES IN IMMUNOCOMPETENT MICE

5 x 10^5 AT3-luc-mCherry BC cells resuspended in 100 µL of PBS 1X were injected into the lateral tail vein of 8–10-week-old C57BL/6 females. Cell migration to the lungs was monitored once a week by intraperitoneal injection of D-luciferin at 150 mg/kg and bioluminescence measurement using an IVIS Spectrum system. Six weeks after cell inoculation, animals were sacrificed, and lungs were collected and photographed under a magnifying microscope. Lung metastases were excised, digested (see below) and cryopreserved until flow cytometry analysis.

BREAST TUMOUR AND LUNG METASTASES DIGESTION

Harvested breast tumours and lung metastases were placed in a Petri Dish for mechanical disaggregation followed by enzymatic digestion in normal medium (DMEM + 2 mM L-Gln + 1 % P/S) supplemented with 0.625 mg/mL collagenase (Sigma-Aldrich, #C9407), 0.125 mg/mL dispase II (Thermo Fisher Scientific, Walthman, Massachusetts, US, #17105041) and 0.1 mg/mL DNase I (Sigma Aldrich, #DN25) for 1-2 h at 37 ºC with shaking (150 rpm). Enzymatic activity was blocked by adding cold blocking medium [normal medium + 10 % FBS + 5 mM ethylenediaminetetraacetic acid (EDTA) (PanReac Applichem, #141952)] and cell suspension was filtered into a new Falcon tube with a 70 µm cell strainer (Corning, #431751). Cells were centrifuged at 500 g for 5' at RT and red blood cells (RBC) were lysed for 15' with a home-made erythrocyte lysis buffer (**Table MM4**). After further centrifugation, cells were resuspended in normal medium + 10 % FBS and filtered again through a 70 µm cell strainer to completely remove undigested fragments. Finally, cells were resuspended in cryopreservation medium and stored at -80 ºC until analysis.

Reagent	Reference	Concentration
Milli-Q H_2O	-	-
Sodium chloride (NaCl)	Sigma-Aldrich, #S9625	138 mM
Potassium chloride (KCl)	Sigma-Aldrich, #49360500	2.7 mM
Tris HCl	PanReac Applichem, #A10865000	5 mM

Reagent	Reference	Concentration
Milli-Q H_2O	-	-
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, #A9434	7.47 g/L
Tris saline buffer	See above	10 %

Table MM4. Erythrocyte lysis buffer recipe. The upper table shows the composition of Tris saline buffer (pH adjusted to 8.0); while the lower table shows the composition of RBC lysis buffer (pH adjusted to 7.2). The buffer was filtered through a 0.22 µm filter unit (Dutchser, #051733) and stored at 4 ºC until use.

SPLENOCYTE ISOLATION

Eight-to-ten-week-old C57BL/6 female mice were euthanised, and spleens were harvested under sterile conditions. The organs were homogenised on a 70 µm cell strainer placed on a 50 mL Falcon tube using the plunger of a syringe and washed with isolation buffer (PBS + 0.1 % BSA + 1 % P/S). After centrifugation at 600 g for 5' at RT, RBC were lysed for 15' followed by another round of centrifugation. Splenocytes were then resuspended in isolation buffer, filtered into a new tube with a 70 µm cell strainer, counted using a Neubauer chamber and used for further procedures or cryopreserved until use.

CANNABINOID TREATMENT OF IMMUNE CELLS AND CO-CULTURES WITH BC CELLS

Isolated splenocytes were activated and/or treated with cannabinoids as indicated in the corresponding Results section. For the activation step, the co-culture medium (RPMI + 2 mM L-Gln + 50 µM βME + 10 % FBS + 1 % P/S) was supplemented with 2.5 µg/mL ConA and 10 ng/mL rmIL-2. At the indicated time, cells were analysed by flow cytometry or pelleted and frozen for further analysis.

For killing assays, a two-dimensional co-culture was performed between C57BL/6-derived splenocytes and the syngeneic TNBC cell line AT3. Briefly, cells were seeded in co-culture medium at different effector-to-target (E:T) ratios in a 24-well plate and were allowed to interact for the indicated time, after which BC cell viability was assessed by crystal violet assay (see below) as an indirect measure of immune cell killing capacity. The ratios used in this thesis were based on the percentage of T cells in the spleen of C57BL/6 females and ratios of E:T previously reported by others (Delirezh et al., 2012; Kanemaru et al., 2022; Lerner et al., 2023).

CELL VIABILITY ASSAYS

AT3 BC cells were seeded in co-culture medium at low density (5 x 10^3 cells/well in a 24-well plate) and co-cultured with immune cells and/or treated with cannabinoids. At the indicated time, cell medium was replaced, and the remaining attached viable cells were stained for 20' at RT with crystal violet solution [0.1 % crystal violet (PanReac Applichem, #2517621606), 20 % methanol (Scharlab, Barcelona, Spain, #ME03022500) in distilled water]. The plate was then thoroughly washed with tap water and allowed to air dry before solubilizing the crystals in methanol for 20' at RT. Absorbance at 570 nm was measured on a RT-6100 microplate reader (Rayto, Shenzhen, China) and was used to relativize the number of viable cells in each condition.

PROTEIN EXTRACTION AND WESTERN BLOT (WB)

As floating cells, splenocytes were collected from the supernatant (see Splenocyte isolation section above) and centrifuged at 600 g for 5' at RT. The cell pellet was then lysed in cold RIPA buffer [100 mM NaCl, 50 mM TrisHCl pH 7.4, 5 mM $MgCl_2$ (Sigma-Aldrich, #M8266), 0.1 % sodium dodecyl sulfate (SDS) (PanReac Applichem, #A25721000), 1 % Triton X-100 (Sigma-Aldrich, #T9284) and 0.5 % deoxycholic acid (Sigma-Aldrich, #30970)] supplemented with protease [2 µg/µL leupeptin (MedChemExpress, Sollentuna, Sweden, #HY-18234A), 2 µg/µL aprotinin (Sigma-Aldrich, #A1153) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, #P7626)] and phosphatase inhibitors [1 mM sodium orthovanadate (PanReac Applichem, #A216)].

Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, California, US, #500-0006) using BSA as standard. 15-25 µg of protein were boiled for 3' at 90 ºC in loading buffer (10 % SDS, 10 mM βME, 0.0125 M Tris-HCl pH 6.8, 0.05 % bromophenol blue, 20 % glycerol) and resolved by SDS-PAGE followed by transfer to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, #1620177) using the Trans-Blot® SD Semi-Dry Transfer Cell System (Bio-Rad). Membranes were blocked with 5 % BSA (w/v) in 0.1 % TBS Tween-20 (TBST) (PanReac Applichem, #142312) for 1 h at RT and incubated overnight (o/n) at 4 ºC with the primary antibody (**Table MM5**) prepared in TBST with 5 % BSA (w/v). Membranes were then washed with TBST and incubated with the corresponding secondary antibody (**Table MM5**) prepared in TBST for 1 h at RT. After a further round of washes, the membranes were incubated with a homemade chemiluminescence reagent [1.25 mM luminol (Sigma-Aldrich, #A8511), 0.25 mM p-coumaric acid (Thermo Scientific, #A15167.14), 100 mM Tris-HCl pH 8.5, in dH_2O] and protein bands were detected on an ImageQuant LAS 500 system (GE HealthCare, Chicago, Illinois, US). Densitometric analysis was performed using the Image Lab™ software (Bio-Rad) and the results were normalized to the expression of the loading control.

Primary antibody	Reactivity	Host	Reference	Dilution
IFNGR1	M	R	CST #84318	1:1000
IFNGR2	H	R	Thermo Scientific #10266-1-AP	1:1000
JAK1	H, M	R	CST #74129	1:1000
pSTAT1 (Tyr701)	H, M	R	CST #9167	1:1000
STAT1	H, M, Rt, Mk	R	CST #9172	1:1000
pSTAT3 (Tyr705)	H, M, Rt, Mk	R	CST #9145	1:1000
STAT3	H, M, Rt, Mk	R	CST #4904	1:1000
PTP1B	H, M, Rt	R	Thermo Scientific #PA5-79894	1:1000
TC-PTP	H, M	R	Thermo Scientific #PA5-78138	1:1000
Vinculin	H, M	M	Sigma-Aldrich #V9264	1:5000
Secondary antibody	**Reactivity**	**Host**	**Reference**	**Dilution**
HRP Mouse IgG	M	S	Cytiva Lifescience #NA931	1:5000
HRP Rabbit IgG	R	D	Cytiva Lifescience #NA934	1:5000
HRP Rabbit IgG	R	G	CST #7074	1:1000

Table MM5. List of antibodies used for Western blot analysis. D: donkey; G: goat; H: human; M: mouse; Mk: monkey; R: rabbit; Rt: rat; S: sheep. HRP: horseradish peroxidase. CST: Cell Signalling Technology. Cytiva Lifescience, Marlborough, Massachusetts, US.

CYTOKINE ARRAY

For the determination of soluble factors in the microenvironment of $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumours, the Proteome Profiler Mouse Cytokine Array Kit (R&D Systems, #ARY006) was employed according to the manufacturer's instructions.

Briefly, tumours were lysed in PBS containing protease (2 µg/µL leupeptin, 2 µg/µL aprotinin and 0.1 mM PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate) and 1 % Triton-X100 prior to centrifugation at 1 x 10^4 g for 5' at 4 ºC. Protein concentration was determined in the supernatant by Bradford assay, using BSA as a standard. 200 µg of each sample were mixed with Array Buffer 4 and detection antibody cocktail and incubated 1 h at RT on a rotary mixer. Parallelly, membranes were blocked with Array Buffer 6 for 1 h at RT with gentle rocking. The sample mixture was then applied to the blocked membrane and incubated o/n at 4 ºC. After three washes with Wash Buffer (10' each), membranes were incubated with diluted streptavidin-HRP for 30' at RT, followed by additional washes. Membranes were finally incubated with Chemi Reagent mix for 1' and exposed on an ImageQuant LAS 500 system. Densitometric analysis was performed using the Image Lab™ software and the results were normalized to the expression of the internal control.

IMMUNOFLUORESCENCE

Prior to immunofluorescence assays, splenocytes were seeded in glass coverslips. Briefly, floating cells were collected from the supernatant (see Splenocyte isolation section above), centrifuged at 600 g for 5' at RT and resuspended in PBS 1X at 1 x 10^6 cells/mL. The cell suspension was then added to the coverslips placed in a 24-well plate and, to promote cell adhesion to the surface, the plate was centrifuged at 150 g for 30' at 4 ºC. Finally, immune cells were fixed in 10 % formalin (PanReac Applichem, #143091.1214) for 10' at RT and stored in PBS 1X at 4 ºC until immunostaining.

For the detection of intracellular granzyme B (GrzB), cells were permeabilised for 10' at RT with permeabilization/wash buffer (PBS + 0.1 % Triton X-100). Cells were then washed three times with the same buffer, blocked for 1 h at RT with blocking buffer (PBS + 0.1 % Triton X-100 + 2 % BSA) and incubated o/n at 4 ºC with fluorochrome-conjugated primary antibody against GrzB (BD Bioscience, San Jose, California, US, #571461) diluted 1:100 in blocking buffer. After three washes, nuclei were stained with 1 µg/mL 4', 6-diamidino-2-phenylindole (DAPI) (Roche Life Science, Basel, Switzerland, #10236276001) for 10' at RT. After a final wash with PBX 1X, coverslips were mounted with Mowiol® mounting medium (Calbiochem, San Diego, California, US, #81381). Fluorescence confocal images were captured using an Olympus FV1200 at the Flow Cytometry and Fluorescence Microscopy Unit (UCM).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

IFN-γ production by immune cells was determined by ELISA performed on 96-well high affinity plates (Thermo Scientific, #439454) according to the protocol provided by the kit manufacturer (Biolegend, San Diego, California, US, #430815). Briefly, supernatant from cultured splenocytes was collected at the indicated time and stored at -80 ºC until use. On the first day of the protocol, a mouse anti-IFN-γ specific rat monoclonal antibody (capture antibody) was coated onto a 96-well plate by o/n incubation at RT. The following day, non-specific binding was blocked by adding Assay Diluent for 1 h at RT with shaking, followed by incubation with standards and samples for 2 h at RT with shaking. Afterwards, a biotinylated rat monoclonal anti-mouse IFN-γ detection antibody was added for 1 h at RT with shaking and Avidin-HRP was subsequently incubated for 30' at RT with shaking. To visualize the reaction, tetramethylbenzidine substrate solution was added, resulting in a blue colour proportional to the concentration of IFN-γ in the sample. Finally, the Stop Solution (4M H_2SO_4, PanReac Applichem, #141058.1611) changed the reaction colour from blue to yellow. Absorbance was measured at 450 nm and corrected at 570 nm using an RT-6100 microplate reader. Between incubations – except for those involving tetramethylbenzidine substrate addition and reaction stopping – the wells were washed three times with wash buffer (PBS + 0.05 % Tween-20).

FLOW CYTOMETRY

Single cells suspensions (from breast tumours and lung metastases digestion, isolated splenocytes or BC cells) were prepared as described in the corresponding Materials and Methods section, washed with FACS buffer (PBS + 2 % FBS + 1 % P/S) and distributed in a V-shape 96-well plate (Deltalab, Barcelona, Spain, #900011.1). To minimize non-specific staining, cells were incubated with Fc blocking reagent (**Table MM7**) for 15' on ice. After centrifugation at 600 g for 5' at 4 ºC, cells were stained for 30' on ice and protected from light with a fluorochrome-conjugated primary antibody cocktail containing a cell viability marker (**Table MM7**). After further centrifugation, cells were resuspended in FACS buffer and transferred to round-bottom polystyrene tubes (Biogen, Cambridge, Massachusetts, US, #9778). Data acquisition was performed in a FACSAria™ III or a FACSymphony A1 system (Becton Dickinson) at the Flow Cytometry and Fluorescence Microscopy Unit (UCM) and data analysis was performed using the FlowJo™ software (BD Bioscience). An example of the gating strategy used in the different analyses can be found in **Figure MM3.**

STATISTICAL ANALYSES

All analyses were performed using GraphPad, Inc. software (San Diego, California, US) and a *p*-value of less than 0.05 was considered statistically significant. Unless otherwise stated, data are expressed as mean ± SEM (standard error of the mean). When 2 independent groups were analysed, an unpaired 2-tailed Student's t-test was used. One-way ANOVA was used to test multi-group comparisons with Tukey's post-hoc analysis. The groups with two-independent variables were tested by 2-way ANOVA. The concentration at which 50 % of the maximum inhibitory effect (IC_{50}) was achieved was calculated by non-linear regression [inhibitor] vs response – variable slope (four parameters). Kaplan-Meier survival curves were statistically compared by the log-rank test.

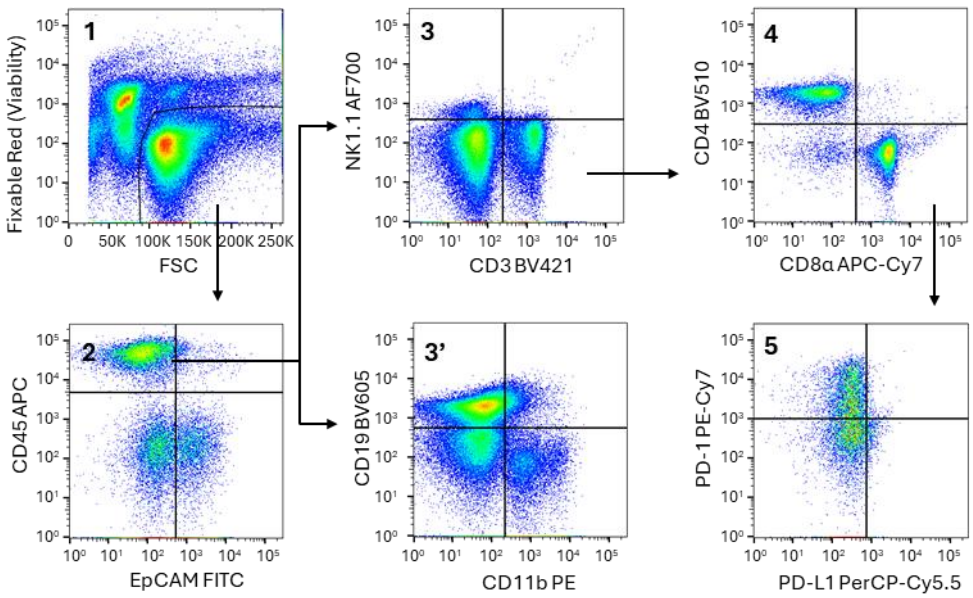

Figure MM3. Gating strategy for the identification of immune cells and their cellular state. (1) Non-viable cells were excluded from the analysis based on their positivity for viability marker dye. (2) Immune cells were selected as $CD45^+$ $EpCAM^-$ and within this gate, subsets of major immune cells were identified (3 and 3'). The main T-cell subsets were then analysed (4), followed by the expression of immune checkpoints (5).

Table MM7. List of antibodies and viability markers used for flow cytometry analysis. PDGFRα: Platelet-derived growth factor receptor α; CAFs: Cancer-associated fibroblasts; NK: Natural killer; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; BV: Brilliant violet; APC: Allophycocyanin; AF: Alexa Fluor®; APC-Cy7: APC-Cyanine7; PE-Cy7: PE-Cyanine7; PerCP-Cy5.5: Peridinin chlorophyll protein-Cyanine5.5.

Antibody	Marker	Fluorochrome	Clone	Reference	Dilution
TruStain FcX™ (Fc blocking)	-		93	BioLegend #101320	1 µg / 10^6 cells
mCD326/Ep-CAM	Tumour cells	FITC	G8.8	BioLegend #118208	1:200
mCD140α/PDGFRα	CAFs	PE	APA5	eBioscience #12-1401-81	1:200
mCD31	Endothelial cells	BV421	MEC13.3	BD Bioscience #562939	1:200
mCD45	Immune cells	APC	30 F11	BD Bioscience #559864	1:200
h/mCD11b	Myeloid cells	PE	M1/70	BioLegend #101208	1:200
mCD19	B cells	BV605	6D5	BioLegend #115540	1:100
mNK1.1	NK cells	AF700	PK136	BioLegend #108730	1:50
mCD3	T cells	B421	17A2	BioLegend #100228	1:40
mCD4	Helper T cells	BV510	GK1.5	BioLegend #100449	1:100
mCD8α	Cytotoxic T cells	APC-Cy7	53/6.7	BioLegend #100714	1:40
mCD279/PD-1	Activation/exhaustion	PE-Cy7	29F.1A12	Biolegend #135216	1:100
mCD274/B7-H1/PD-L1	Immunosuppression	PerCP-Cy5.5	10F.9G2	BioLegend #124334	1:40
mCD69	Activation	PE-Cy7	H1.2F3	BD Bioscience #552879	1:80
mCD107α	Cytotoxicity	PE	1D4B	BD Bioscience #558661	1:80
mH2-Class I	Antigen presentation	R718	M1/42	BD Bioscience #751875	1:100
Fixable Viability Dye eFluor™ 780	Viability	-	-	Thermo Scientific #65-0865-14	1:1000
LIVE/DEAD™ Fixable Red	Viability	-	-	Thermo Scientific #L34971	1:600

RESULTS

AIM 1. CHARACTERISATION OF CB_2R EXPRESSION IN IMMUNE CELLS

The concept of cancer has evolved from a tumour cell-centric model to a TME-centric paradigm, highlighting the importance of diverse non-malignant components in cancer biology. Despite advances in elucidating the complex interactions among molecular and cellular components within the TME, the specific role of the ECS in this context remains poorly understood. To address this knowledge gap, we focused our study on CB_2R, due to its high expression in immune cells and its well-stablished immunomodulatory functions. Given the pivotal role of the immune system in cancer pathophysiology, elucidating the function of CB_2R within the breast tumour immune microenvironment (BTIME) may yield novel insights into the mechanisms underlying initiation and progression of this disease.

IN SILICO ANALYSIS OF CB_2R EXPRESSION IN NON-TRANSFORMED TISSUES AND IMMUNE CELLS

To assess the role of CB_2R in the BTME, we first characterised its physiological mRNA expression in non-transformed tissues and cells by analysing publicly available databases. Bulk tissue RNA-sequencing (RNA-seq) data from the Human Protein Atlas project (HPA) (Uhlén et al., 2015) showed that *Cnr2* was enriched in lymphoid tissues (such as lymph node, spleen or tonsil), with virtually no expression in organs with comparatively low immune cell content like the brain or the parathyroid gland (**Figure R1**).

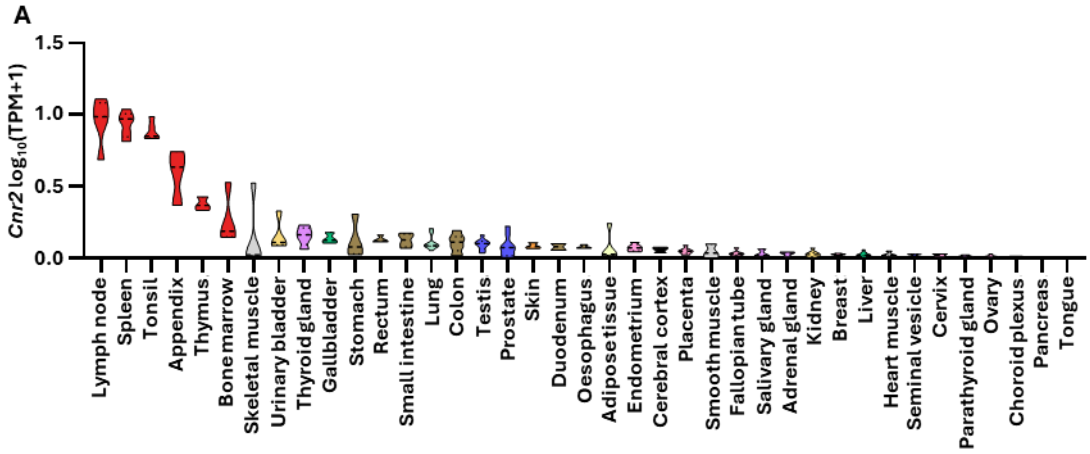

Figure R1. Bulk human CB_2R gene (*Cnr2*) expression. Data were obtained from the HPA project. Tissues with common functional features are represented with the same colour. Expression values are expressed as $\log_{10}$(TPM+1), where TPM is transcripts per million.

Similar results were observed in an independent RNA expression dataset of human postmortem samples from the Genotype-Tissue Expression (GTEx) project (Lonsdale et al., 2013): *Cnr2* expression was higher in immune-related tissues including spleen, small

intestine and blood, whereas it was almost undetectable in structures with a lower presence of immune cells such as the heart or the female reproductive system (**Figure R2**).

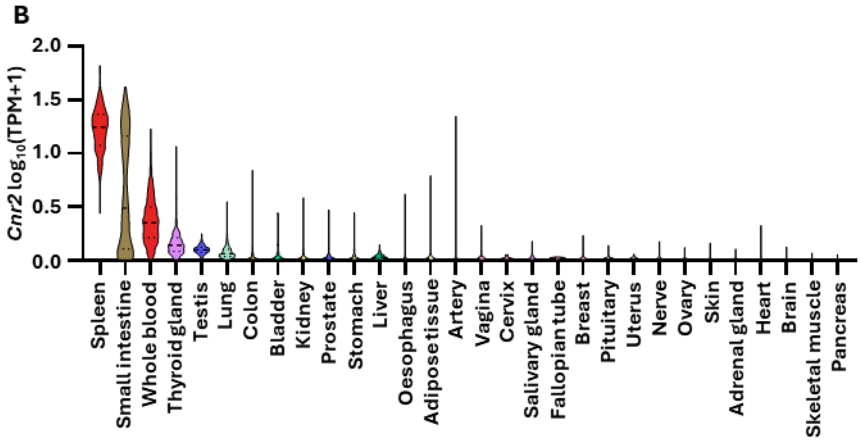

Figure R2. Bulk human CB_2R gene (*Cnr2*) expression. Data were obtained from the GTEx project. Tissues with common functional features are represented with the same colour. Expression values are expressed as $\log_{10}$(TPM+1), where TPM is transcripts per million.

Based on these results, we further assessed *Cnr2* expression pattern specifically in immune cells. HPA data showed high expression in polymorphonuclear cells (PMN, mainly basophils and eosinophils), closely followed by lymphocytes, including B and Natural killer (NK) cells (**Figure R3A**). In contrast, expression was nearly absent in myeloid cells like myeloid dendritic cells (mDC) or monocytes (MO). A second RNA-seq dataset comprising 29 human immune cell types and peripheral blood mononuclear cells (PBMCs) confirmed these observations: basophils emerged as the principal CB_2R-expressing population, followed by B and NK cells, while MO displayed the lowest expression levels (**Figure R3B**).

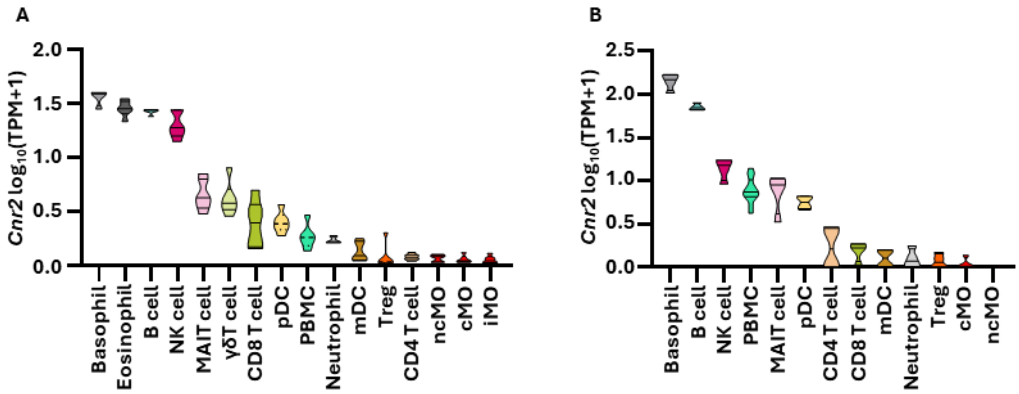

Figure R3. *Cnr2* expression in human immune cells. Data were obtained from (A) the HPA project and (B) the RNA-seq published by Monaco et al., 2019 (accession number: GSE107011). Data were transformed to $\log_{10}$(TPM+1) for graphical representation. cMO: classical monocytes; iMO: intermediate monocytes; MAIT: mucosal-associated invariant T cells; mDC: myeloid dendritic cells; ncMO: non-classical monocytes; PBMC: peripheral blood mononuclear cells; pDC: plasmacytoid dendritic cells; Treg: regulatory T cells.

The expression of the cannabinoid receptor in immune cells appeared to be conserved between human and mice, as evidenced by the analysis of murine sample databases. RNA-seq profiles of immune cells from unchallenged mice revealed that B cells exhibited the highest expression of CB_2R, whereas plasmacytoid dendritic cells (pDC) and macrophages (MF) showed minimal expression (**Figure R4A**). These results were further validated through the analysis of a second gene expression microarray dataset of multiple immune lineages isolated from 6-week-old C57BL/6 male mice: *Cnr2* was again predominantly expressed in cells of the lymphoid linage, including B and CD8 cytotoxic T cells, while pDCs and MFs displayed the lowest levels (**Figure R4B**). Interestingly, both datasets included NK and NKT cells, revealing significantly different *Cnr2* levels in each population ($p = 0.0003$ and $p < 0.0001$ NKT vs NK, respectively).

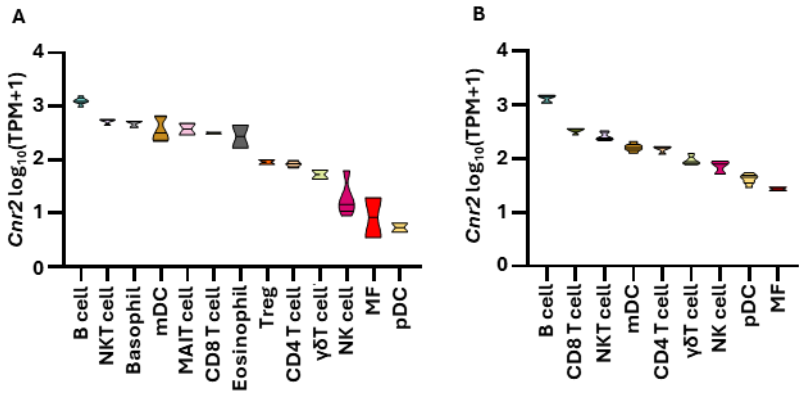

Figure R4. *Cnr2* expression in mouse immune cells. Data were obtained from (A) the RNA-seq published by Yoshida et al., 2019 (accession number: GSE109125) and (B) a microarray generated as part of the Immunological Genome Project (ImmGen) (accession number: GSE15907). Data were transformed to $\log_{10}$(TPM+1) for graphical representation. MF: macrophages; γδT: gamma-delta T cells; NKT: Natural killer T cells.

Regarding CB_2R expression in the breast, *Cnr2* was virtually undetectable in human non-transformed tissue (**Figures R1 and R2**). This is in line with previous work from our group that showed no CB_2R protein presence in non-tumour breast tissue (Caffarel et al., 2006, 2010; Pérez-Gómez et al., 2015). Since all these studies analysed the bulk tissue and had no resolution at the cellular level, they suggested that the main cellular constituents of the breast (i.e., epithelial cells) do not express this receptor. However, and although in a small percentage compared to mammary epithelial cells, this organ contains other types of cells that may have significant amounts of CB_2R, being immune cells among them. Supporting this notion, single-cell RNA-seq (scRNA-seq) profiling of the human mammary gland revealed significant levels of CB_2R in non-epithelial mammary cells. Specifically, clusters of T cells (c-0, c-2, c-10, c-15 and c-24) were the major CB_2R-expressing population, followed by macrophages (c-3, c-18 and c-19) and fibroblasts (c-5, c-12 and c-13). In contrast, CB_2R mRNA was barely detected in other stromal populations such as endothelial cells (c-22) or adipocytes (c-6, c-7, c-8 and c-23) (**Figure R5**).

Regarding T cells, and paralleling the observations made in isolated immune cells, CB_2R mRNA was predominantly expressed within clusters corresponding to NKT (c-15: *GZMB, KIR2DL4*) and cytotoxic T cells (c-2: *CD3ε, CD8A*). Additional CB_2R-positive cells were identified within a special group of regulatory T cells exhibiting inflammatory characteristics

(c-0: *CD3ε, FOXP3, IL17A*), as well as among biphenotypic lymphocytes, or double expresser cells, which simultaneously express both B and T cell markers (c-10: *MS4A1, CD3ε, MBP*) (**Figure R5**).

Among macrophage populations, the cluster displaying the highest CB_2R expression corresponded to a biphenotypic B cell/macrophage population (c-3), whereas cluster c-19 was composed of anti-inflammatory, M2-like macrophages (**Figure R5**).

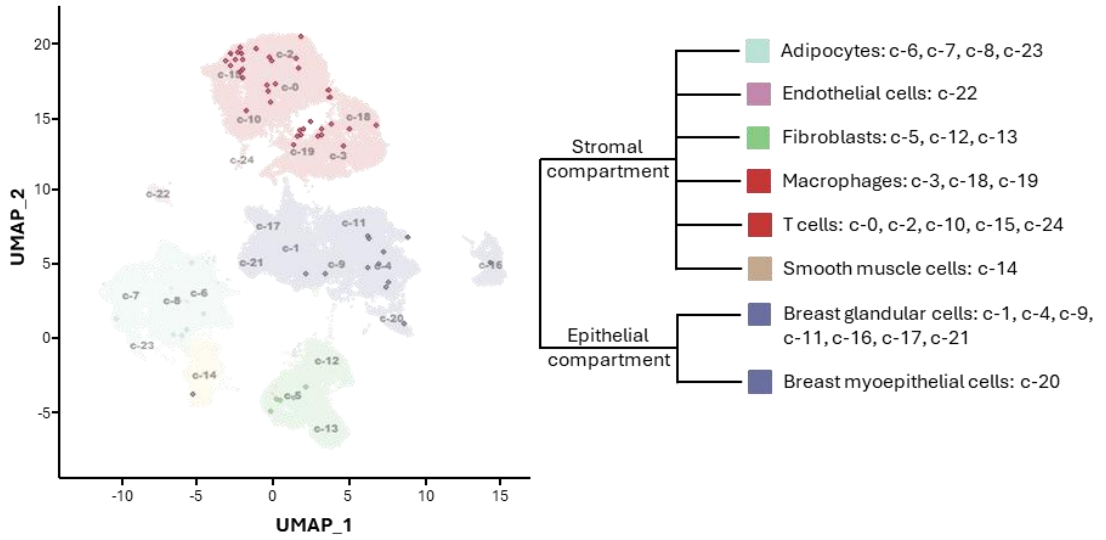

Figure R5. scRNA-seq of CB_2R mRNA expression in the human non-transformed breast. Putative cell clusters of adult human breast populations, defined by scRNA-seq data from the HPA project, are depicted on the left and annotated on the right. Dark-coloured dots on the left indicate cells expressing CB_2R. UMAP-1 and UMAP-2 represent the two main dimensions of the Uniform Manifold Approximation and Projection (UMAP) used to visualise cell clusters based on transcriptomic similarity.

In view of these results and given the importance of cytotoxic T cells in antitumour immunity, we set out to investigate how CB_2R levels vary through the lifespan of this cell type, focusing on the processes of activation and differentiation into effector and memory subsets.

Transcriptomic data from human CD8 T cells—comparing naive (i.e., those that have not encountered cognate antigen) and activated cells (stimulated *ex vivo* for 4 h with α-CD3 and α-CD28 antibodies)—revealed a significant reduction in CB_2R expression following activation (**Figure R6A**). A second expression profiling by high throughput sequencing allowed a more detailed characterisation by analysing isolated human cytotoxic T cells stimulated with anti-CD3/CD28 beads for seven days with RNA extracted at the indicated time-points (**Figure R6B**): the expression of CB_2R decreased in a time-dependent manner, reaching minimum levels after 48 h. Interestingly, the baseline levels were recovered and even exceeded after prolonged exposure to the activating stimulus, which can lead to differentiation into memory T cells.

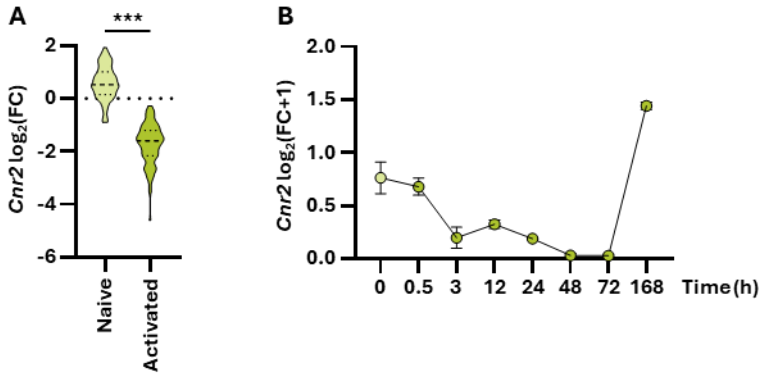

Figure R6. CB_2R mRNA expression in isolated human CD8 T cells. Data were obtained from (A) the Database of Immune Cell Expression (DICE) project established by Schmiedel et al., 2018 and (B) the RNA-seq published by Lui et al., 2024 (accession number: GSE247647). Student's t-test: *** < *p* 0.001 vs naive.

Additionally, we analysed two murine datasets in which T cells were isolated from the spleens of C57BL/6 animals infected with either lymphocytic choriomeningitis virus (LCMV) (**Figure R7A**) or *Listeria monocytogenes* overexpressing the ovalbumin peptide (LisOVA) (**Figure R7B**) at the indicated time points. Both represent acute infection protocols, wherein pathogen levels are low or undetectable in the spleen by day 15 post-infection (Pope et al., 2001), thereby allowing the generation of memory T cells. Results analogous to those observed in human cells were observed in these analyses: *Cnr2* expression in cytotoxic T cells was the highest in the naive state (i.e., 0 d), decreased upon activation and recovered in the memory subsets. Interestingly, Yoshida and collaborators differentiated an effector-to-memory cell transition state (memory precursor, MP) that revealed CB_2R levels in between the two stages (**Figure R7A**). This state may correspond to the 15-day activation period reported by the ImmGen project, in which CB_2R expression began to recover after reaching minimal levels (**Figure R7B**).

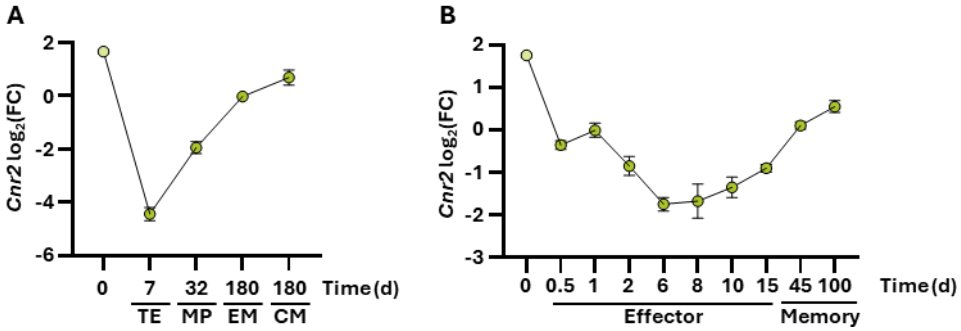

Figure R7. Mouse CB_2R mRNA expression in isolated CD8 T cells. Data were obtained from (A) the RNA-seq published by Yoshida et al., 2019 (accession number: GSE109125) and (B) the microarray generated as part of the ImmGen project (accession number: GSE15907). CM: central memory; EM: effector memory; MP: memory precursor; TE: terminal effector.

Collectively, our data provide a detailed characterisation of CB_2R expression in immune cells and suggests, as previously reported by others, that this receptor may play a significant immunomodulatory role, particularly in regulating the effector functions of cytotoxic T cells in both humans and mice.

IN VIVO ANALYSIS OF CB_2R EXPRESSION

Next, we further characterised CB_2R expression in *in vivo* settings. To this aim, we utilized the C57BL/6 CB_2R^{eGFP} mouse (López et al., 2018), in which the expression of the reporter gene (enhanced Green Fluorescent Protein, eGFP) was under the control of the endogenous *Cnr2* promoter. Thus, only cells expressing CB_2R would be eGFP-positive (**Figure R8A**). In addition, and to analyse the expression of CB_2R specifically in the BTIME, we generated triple negative breast tumours in these mice by orthotopic injection of the syngeneic AT3 cell line and harvested them at different time points (**Figures R8B and R8C**). Importantly, this model offers the advantage of allowing us to specifically examine the contribution of CB_2R-expressing (GFP^+) and CB_2R-lacking (GFP^-) cells to the overall tumour phenotype, particularly with regard to immunosurveillance marker expression.

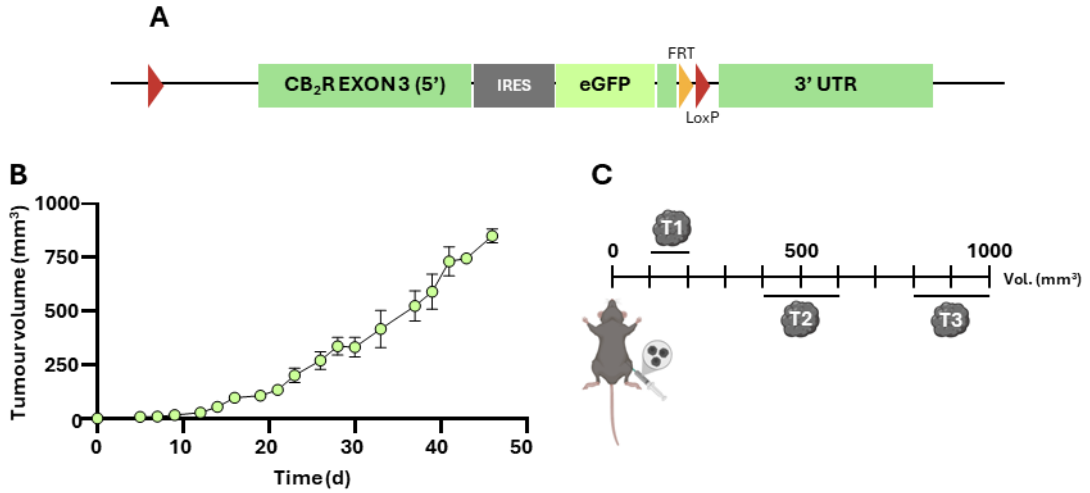

Figure R8. Outline of the use of the C57BL/6 CB_2R^{eGFP} mouse model. (A) Schematic representation of the Cnr2-eGFP allele. (B) Growth of tumours generated by orthotopic injection of AT3 cells. (C) Experimental set up for the generation of TNB tumours and sample collection. Tumours were harvested at three different timepoints, defined according to tumour volume (Vol.): 100-200 (T1), 400-600 (T2) and 800-1000 (T3) mm^3.

First, we conducted a comparative characterisation of the splenic immune cell composition in naive (non-tumour bearing) and tumour-bearing females. In naive animals, B cells were the major population, while NK and NKT lymphocytes were virtually undetectable. Regarding to T cells, the spleens contained a higher number of CD4 helper T cells than CD8 cytotoxic T cells. Interestingly, the spleens from tumour-bearing mice revealed significant changes in their immune cell profile: while the percentage of T cells decreased, the proportion of all other immune cells increased. These changes also occurred at T cell level: CD4 T cells augmented, while CD8 T cells diminished (**Figure R9A**).

Regarding CB_2R expression in the different splenic immune cell populations, naive female mice showed a pattern that closely resembled that observed in the *in silico* studies: it was higher in B and CD8 T cells and lower in NK and CD4 T cells. Myeloid cells exhibited intermediate levels, likely reflecting heterogeneous receptor expression among the various subpopulations within this group (the use of the CD11b pan-marker did not allow differentiation between mDC, pDC and MF) (**Figures R9B and R9C**).

Consistent with the *in silico* findings, an activating stimulus (a tumour in this case) decreased CB_2R levels in the total pool of immune cells and specifically in the populations that presented higher CB_2R expression in naive animals (**Figures R9B and R9C**).

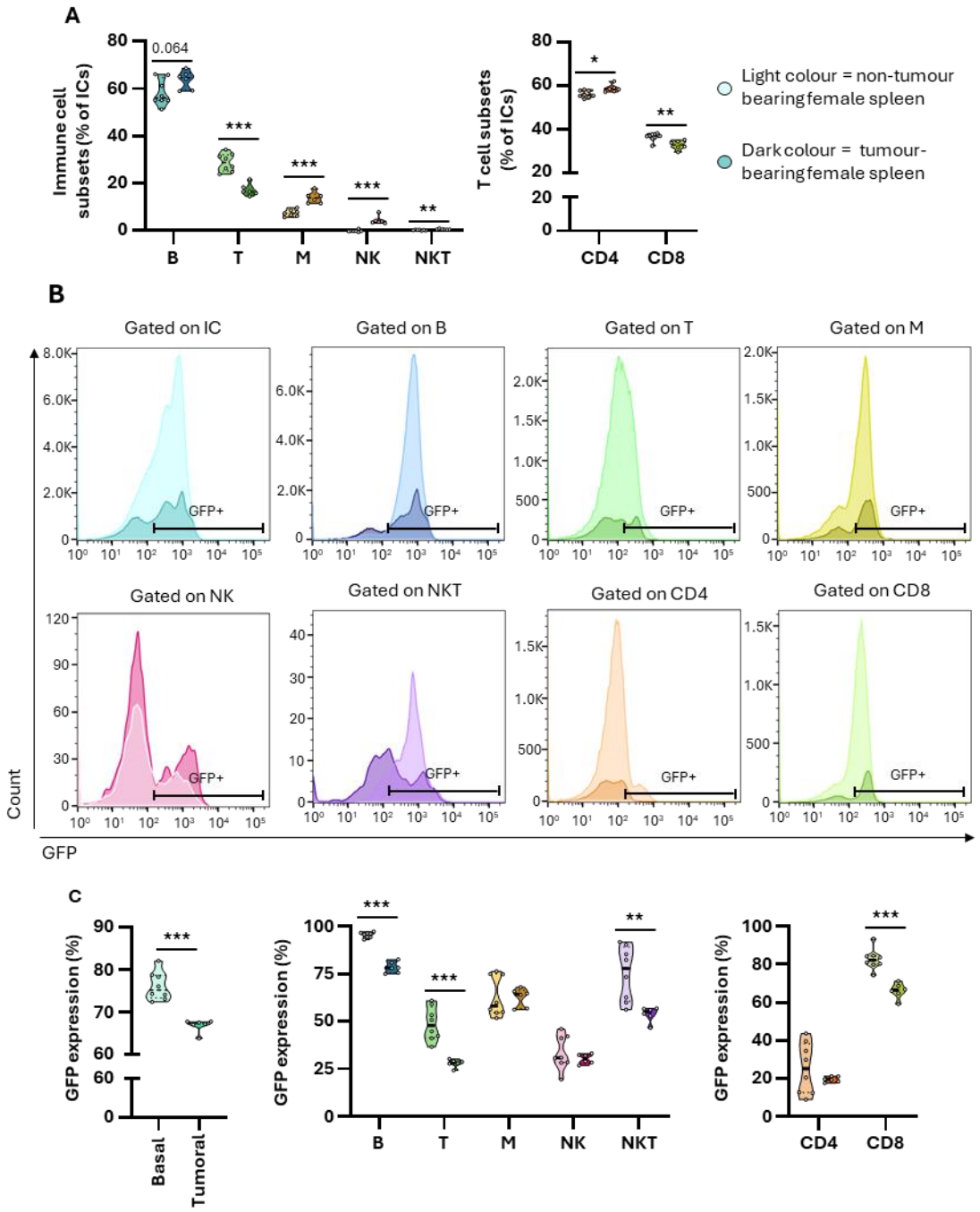

Figure R9. Analysis of the splenic cell composition and CB_2R expression in naive and tumour-bearing female mice. (A) Characterisation of the cellular composition of spleens from naive and tumour-bearing (T1-T2) female mice. (B) Representative flow cytometry plots showing GFP (CB_2R) expression in splenocytes from naive and tumour-bearing females. (C) Quantification of GFP (CB_2R) expression from B in total immune cells (left panel) and the main immune and T cell subsets (middle and right panels, respectively). For each population analysed, immune cells isolated from non-tumour bearing mice are shown on the left in light colours, while those from tumour-bearing mice are depicted on the right in dark colours. M: myeloid cells. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs naive.

Once CB_2R expression was characterised in the spleen, we analysed its presence in the BTIME. To this end, we used the animal model described above (AT3 cells orthotopically injected into C57BL/6 CB_2R^{eGFP} females, and tumours harvested at different time points, **Figure R8**).

We first assessed the general stromal composition of late-stage tumours (T3) and found that immune cells were the main tumour-infiltrating population (45 %), with fibroblasts (CAFs) and endothelial cells (ECs) accounting for less than 10 % (**Figure R10A**). All three cell types exhibited high levels of CB_2R, with CAFs showing the highest expression (**Figures R10B and R10C**).

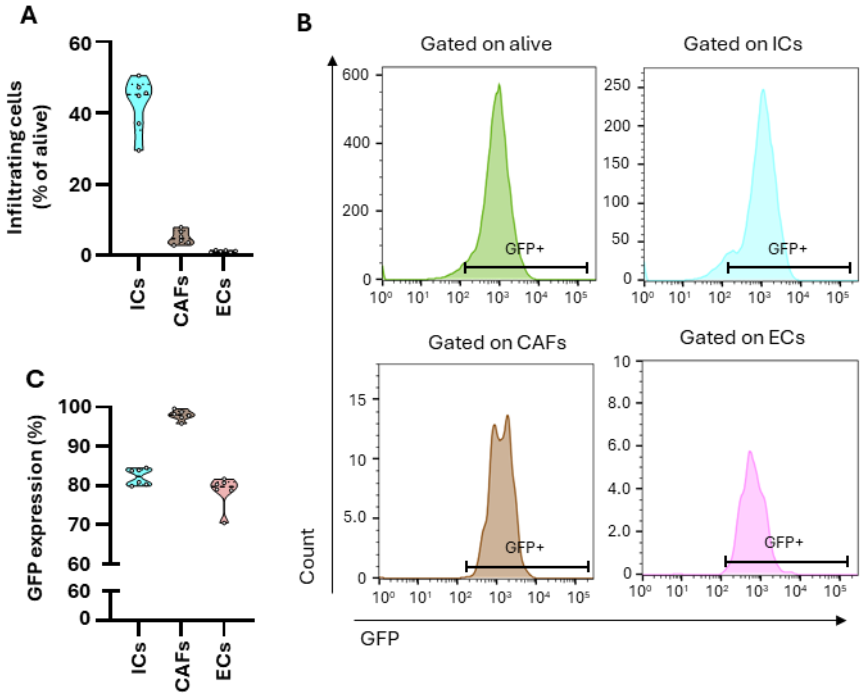

Figure R10. Characterisation of the stromal cell composition of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. (A) Percentage of immune cells (ICs), cancer-associated fibroblasts (CAFs) and endothelial cells (ECs) in tumours harvested at T3. (B) Representative flow cytometry plots showing CB_2R expression in different stromal cell populations. (C) Quantification of GFP (CB_2R) expression calculated from panel B.

When focusing specifically on the immune infiltration and how it evolved over time, we observed a marked decrease in total immune cell infiltration as tumours increased their size, suggesting a transition towards immune exclusion. When dissecting this analysis focusing on the different immune cell subsets, we only observed a decrease in myeloid cells that could justify the observed decrease in immune cell infiltration (**Figure R11**). In addition, and although the percentage of T cells remained unchanged with time, the CD4 T cell subset significantly decreased in parallel to tumour growth (**Figure R11**). On the contrary, CD8 T cells became more prevalent in late-stage tumours. This shift resulted in an inverted CD4/CD8 ratio, which dropped below 1 in the advanced stage (T1: 7.13 ± 0.91; T2: 2.69 ± 0.99; T3: 0.16 ± 0.06).

Regarding other cell types, no changes were observed in NKTs, whereas the proportion of NK and B cells increased with tumour size (**Figure R11**).

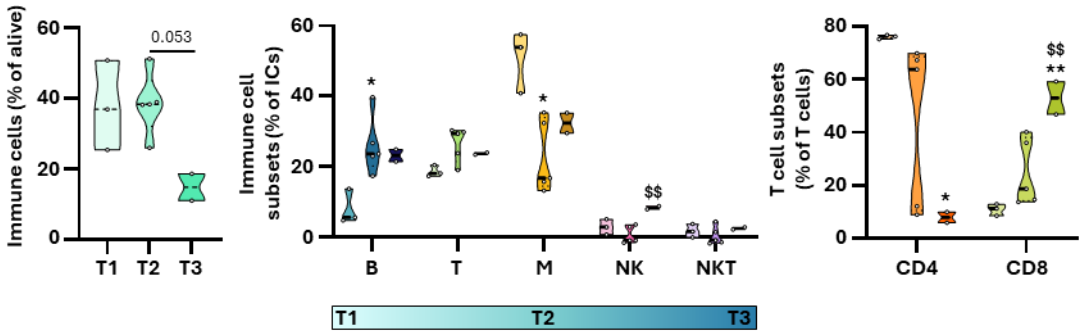

Figure R11. Characterisation of the TIME in TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. Infiltration of total immune cells (left panel), major immune subsets (middle panel) and T cell subpopulations (right panel) in the tumour stroma. The darker the colour, the higher the tumour volume (from left to right: T1-T2-T3). One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ vs T1; $$ $p < 0.01$ vs T2.

After analysing the immune composition of the tumours, we proceeded to evaluate CB_2R expression within the different immune cell populations across progression. No significant changes in CB_2R levels were observed either in the total pool of immune cells or in most of the individual cell types analysed, except for NKT cells, which exhibited a significant decrease in its expression at T3 (**Figure R12**).

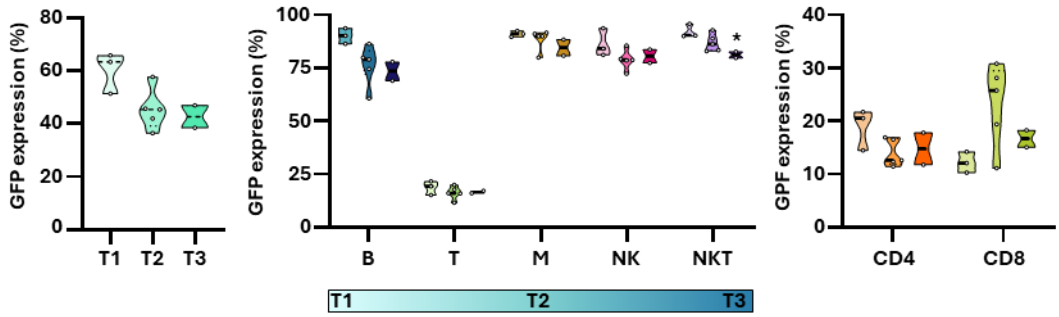

Figure R12. CB_2R expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. GFP (CB_2R) expression in total immune cells (left panel), major immune subsets (middle panel) and T cell subpopulations (right panel), as assessed by flow cytometry. The darker the colour, the higher the tumour volume (from left to right: T1-T2-T3). One-way ANOVA: * $p < 0.05$ vs T1.

Interestingly, when comparing CB_2R expression in tumour-infiltrating immune cells to their counterparts isolated from the spleen of non-tumour bearing females (**Figure R9C**), we observed several significant differences (**Table R1**). Specifically, tumour-infiltrating B and T cells, including both subsets (CD4 and CD8), expressed significantly lower levels of CB_2R than their naive counterparts, whereas the opposite was observed for myeloid, NK and NKT cells, which showed higher CB_2R expression in the tumour-infiltrating populations compared to naive cells. Further studies will be required to determine whether these changes are a cause or a consequence of the TME, and to elucidate the functional consequences of such modulation.

Cell population	Mean ± SEM CB_2R expresión (%)		*p* value
	Naive splenic ICs	Tumour-infiltrating ICs	
B	95.58 ± 0.498	79.74 ± 3.171	0.0004 (***)
T	48.87 ± 3.132	17.19 ± 0.902	< 0.0001 (***)
M	63.15 ± 3.740	88.46 ± 1.406	< 0.0001 (***)
NK	33.61 ± 3.175	81.43 ± 1.830	< 0.0001 (***)
NKT	75.41 ± 5.036	87.28 ± 1.616	0.0257 (*)
CD4	25.33 ± 4.735	15.62 ± 1.165	0.0424 (*)
CD8	82.65 ± 1.960	18.52 ± 2.343	< 0.0001 (***)

Table R1. Comparison of CB_2R expression in naive splenic immune cells and tumour-infiltrating immune cells. For tumour-infiltrating immune cells, data from all three tumour volumes were pooled, as no significant differences were detected among them. Student't t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs naive splenic ICs.

Although both the proportion of infiltrating immune cells (Kurozumi et al., 2019) and CB_2R expression within the TME (Gruber et al., 2021; X. Xiong et al., 2022) have been correlated with clinicopathological variables, inconsistencies among studies underscores the need for a more comprehensive characterisation of the functional heterogeneity of cells within the TME. Due to the relevance of the PD-1/PD-L1 (programmed cell death protein 1 / programmed death ligand-1) axis in immune evasion, we next analysed the expression of these two proteins in our animal model.

PD-1 expression did not significantly change neither in the total immune cell population (**Figure R13A**) or specifically in CD8 T cells (**Figure R13B**) when considering all tumour stages. However, this apparent lack of significance may be attributable to the variability of expression observed at T2. Notably, when comparing T1 and T3 tumours directly, a statistically significant increase in PD-1 expression was detected ($p = 0.038$ for ICs and $p = 0.025$ for CD8 T cells). These findings could reflect a state of T cell exhaustion, potentially contributing to tumour progression through impaired antitumour immunity.

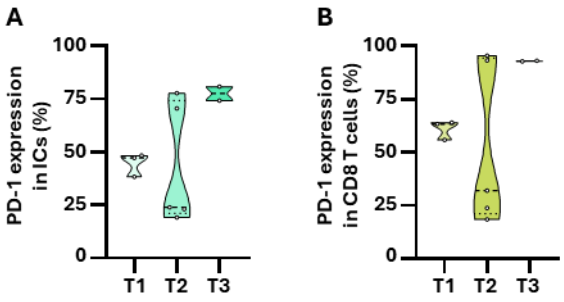

Figure R13. PD-1 expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. PD-1 expression in the total pool of immune cells (A) and in cytotoxic T cells (B), as assessed by flow cytometry. One-way ANOVA vs T1.

Interestingly, when PD-1 expression was studied in more detail, we observed significant differences between GFP^+ (CB_2R^+) and GFP^- (CB_2R^-) immune cells. First, PD-1 expression did not significantly change as tumours grew in both CB_2R^+ and CB_2R^- cells when considering all tumour stages (**Figure R14**). Second, PD-1 levels were higher in immune cells lacking

CB_2R than in cells expressing this receptor (**Figure R14A**). Similar observations (higher PD-1 expression in CB_2R^- vs CB_2R^+ cells) were made in cytotoxic T cells, but only in early stage (T1) tumours (**Figure R14B**). These results suggest that CB_2R may act by raising the threshold required for the initial activation of CD8 T cells; however, this regulatory effect appears to be overridden by other dominant immunosuppressive signals as tumours grow.

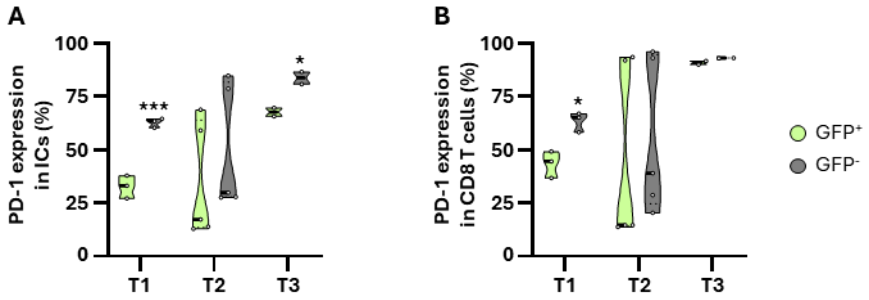

Figure R14. PD-1 expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice, stratified according to CB_2R expression. PD-1 expression in GFP^+ (CB_2R^+) or GFP^- (CB_2R^-) total immune cells (A) and in cytotoxic T cells (B), as assessed by flow cytometry. Student t-test: * $p < 0.05$, *** $p < 0.001$ vs GFP^+.

In our mouse model, we observed that tumour-infiltrating CD8 T cells expressed significantly lower levels of CB_2R compared to cytotoxic T cells isolated from the spleen of non-tumour bearing female mice (**Table R1**). As inferred from the *in silico* analyses of CB_2R expression following CD8 T cell stimulation (**Figure R6 and R7**), this reduction may be indicative of an activated phenotype. However, further analysis of an RNA-seq dataset examining genes involved in cytotoxic T cell exhaustion suggested instead that the low CB_2R levels at T2 and T3 were linked to a dysfunctional phenotype caused by chronic antigen exposure (a tumour in this case) (**Figure R15A**), consistent with the sustained high PD-1 expression detected in this context (**Figure R15B**). This interpretation was further supported by our observation of increased PD-1 expression in CD8 T cell from larger tumours compared to smaller ones (**Figure R13**). Additionally, these transcriptomic data also provided a plausible explanation for the lower expression of PD-1 in CB_2R-expressing T cells compared to CB_2R-lacking ones at T1 (**Figure R14B**): following an activating stimulus, CB_2R is downregulated whereas PD-1 is induced, therefore suggesting that CB_2R-lacking T cells may be more readily activated.

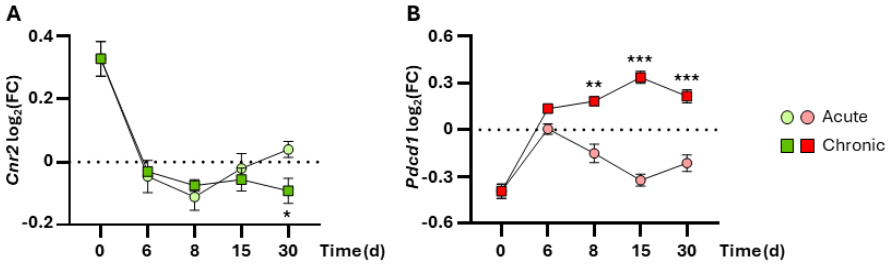

Figure R15. CB_2R and PD-1 mRNA expression in mouse CD8 T cells following acute or chronic antigen stimulation. (A-B) Changes in *Cnr2* (A) and *Pdcd1* (B) mRNA expression in isolated cytotoxic T cells under acute pr chronic activation. Data were obtained from the RNA-seq dataset published by Doering et al., 2012 (accession number: GSE41867) and are presented as to log_2(FC), where FC was fold change. Light-coloured circles represent acute stimulation, while dark-coloured squares indicate chronic stimulation. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs respective acute timepoint.

PD-1's ligand, PD-L1, in addition to being highly expressed on tumour cells as an immune evasion mechanism, can also be expressed by immune cells within the TME, particularly antigen-presenting cells (APCs) and T cells. In our model, we observed a general trend toward decreased PD-L1 expression as tumour size increased (p = 0.0523 T2 vs T1) (**Figure R16A**), an expression pattern that reached statistical significance when specifically analysing CD4 T cells (**Figure R16B**). In contrast, no changes in PD-L1 levels in myeloid cells—the main APCs—were observed in advanced tumours compared to early stages (**Figure R16C**). These results are particularly noteworthy as tumour progression has traditionally been linked to increased PD-L1 levels. However, most of the studies do not distinguish the specific contribution of tumour and non-tumour cells to overall PD-L1 expression, and it has been reported that PD-L1 levels in different populations of the TME may even have opposite prognostic implications (Kim et al., 2016). Considering our results, we hypothesise that the transition towards a more immunosuppressive microenvironment may dampen PD-L1-inducing expression signals in helper T cells, whereas PD-L1 expression in myeloid cells, once established, would be more stable and independent of these factors.

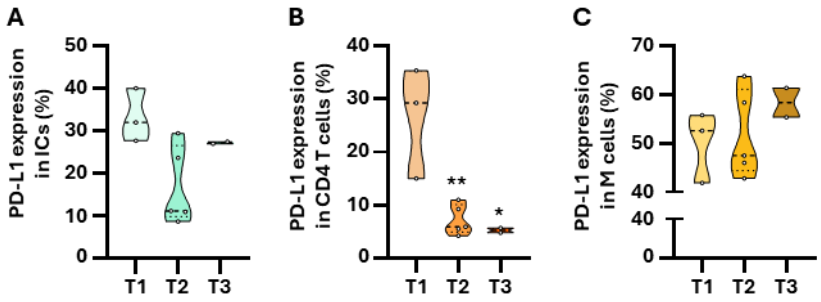

Figure R16. PD-L1 expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. PD-L1 expression in the total pool of immune cells (A), helper T cells (B) and myeloid cells (C), as assessed by flow cytometry. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ vs T1.

As for PD-1, striking differences in PD-L1 expression were observed when the analysis was made discriminating GFP^+ (CB_2R^+) and GFP^- (CB_2R^-) cells. In this case, cell populations lacking CB_2R presented virtually undetectable levels of PD-L1 in all tumour stages. In contrast, CB_2R-positive cells expressed high levels of PD-L1 in all cell types analysed (total immune cells, CD4 T cells and myeloid cells) (**Figure R17**). In addition, and although PD-L1 expression remained stable in the total immune pool and in myeloid cells (**Figures R17A and R17C**), it significantly decreased in helper T cells as tumours progressed (**Figure R17B**). These results suggest that CB_2R may promote an immunosuppressive microenvironment—probably by favouring the differentiation of helper T cells into Tregs, and sustaining an immunoregulatory phenotype in myeloid cells—potentially contributing to tumour immune evasion.

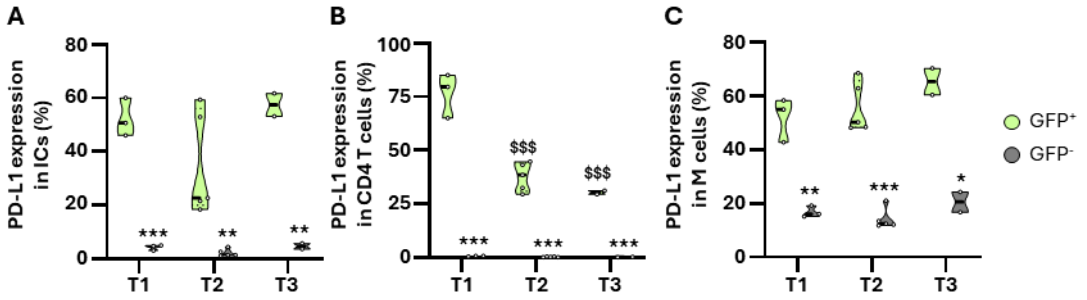

Figure R17. PD-L1 expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice, stratified according to CB_2R expression. PD-L1 expression in GFP^+ (CB_2R^+) or GFP^- (CB_2R^-) total of immune cells (A), helper T cells (B) and myeloid cells (C), as assessed by flow cytometry. Student t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs GFP^+. One-way ANOVA: $$$ $p < 0.001$ vs T1.

To further investigate the role of CB_2R in antitumour immunity, we next analysed the functional state of tumour-infiltrating immune cells. Specifically, we used CD69 and CD107α as markers of early activation and degranulation, respectively. Due to technical limitations, these analyses could only be performed in T2 and T3 tumours, thereby losing information regarding the immune response in small tumours. This, together with the small sample size, calls for caution when interpreting the results, and warrants future experiments to validate them.

Regarding CD69, we observed a significant increase in its expression in larger tumours when total immune cells were analysed (**Figure R18A**). However, we were not able to identify the specific cell population responsible for this increase, as all cell types studied (T, NK and NKT cells) (**Figures R18B, R18C and R18D**, respectively), presented very similar CD69 levels regardless of tumour size. These results could reflect an overall ongoing immune response, but also the emergence of immune dysfunction within the BTME.

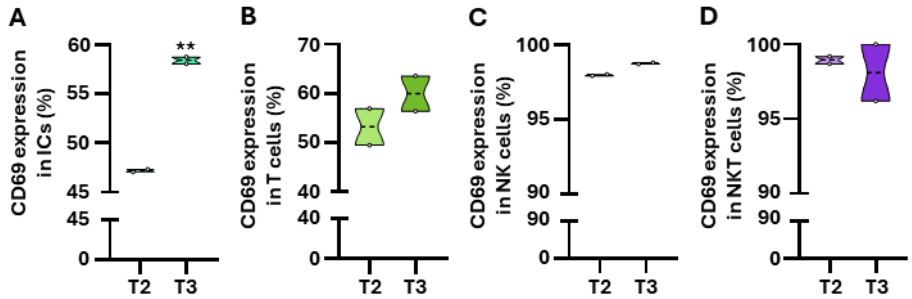

Figure R18. CD69 expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. CD69 expression in the total pool of immune cells (A), T cells (B), NK cells (C) and NKT cells (D) was assessed by flow cytometry. Student t-test: ** $p < 0.01$ vs T2.

When we separately analysed GFP^+ (CB_2R^+) and GFP^- (CB_2R^-) immune cells, we observed increased CD69 expression in larger tumours in both CB_2R-expressing and CB_2R-lacking cells, although this increase was less pronounced in the latter, probably due to their higher baseline levels (**Figure R19A**). However, we could not unequivocally identify the specific cell population(s) accounting for this pattern, as only a modest increase in CD69 expression

was detected in NK cells expressing CB_2R (**Figure R19C**). On the contrary, no significant differences in CD69 levels were found in T cells or NKT cells, regardless of CB_2R-expression and tumour size (**Figure R19B and R19D**, respectively). These results suggest that CB_2R does not significantly influence immune activation or dysfunction, at least within the principal immune subsets analysed.

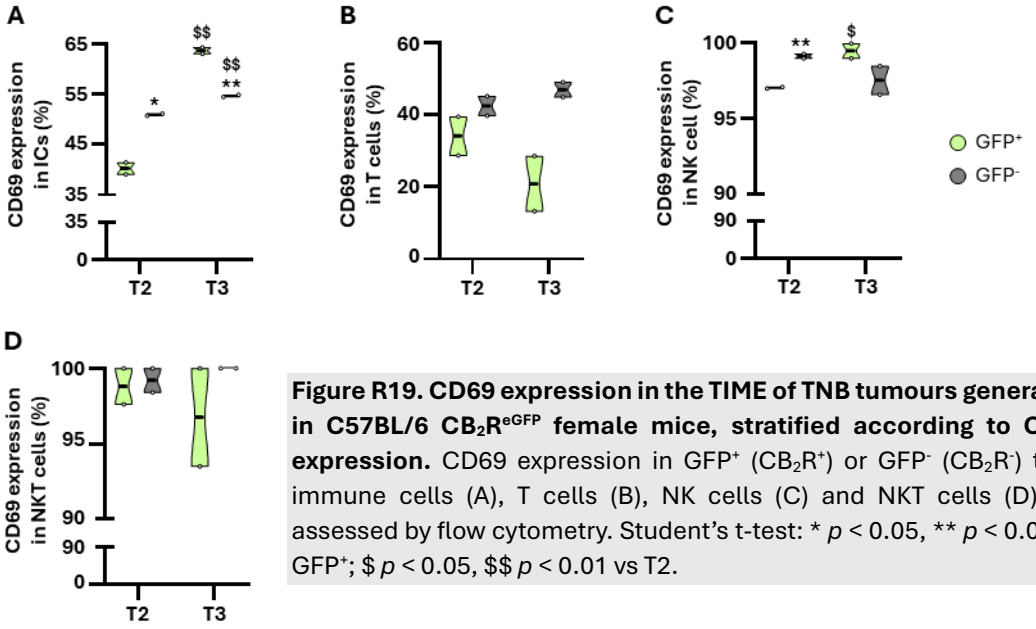

Figure R19. CD69 expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice, stratified according to CB_2R expression. CD69 expression in GFP^+ (CB_2R^+) or GFP^- (CB_2R^-) total immune cells (A), T cells (B), NK cells (C) and NKT cells (D), as assessed by flow cytometry. Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs GFP^+; \$ $p < 0.05$, \$\$ $p < 0.01$ vs T2.

As for the degranulation marker CD107α, no differences were observed between T2 and T3 either in the total immune cell pool or within the subpopulations studied (**Figure R20**). These results suggest that, despite the progression toward a more immunosuppressive microenvironment, immune cells may retain their cytotoxic capacity.

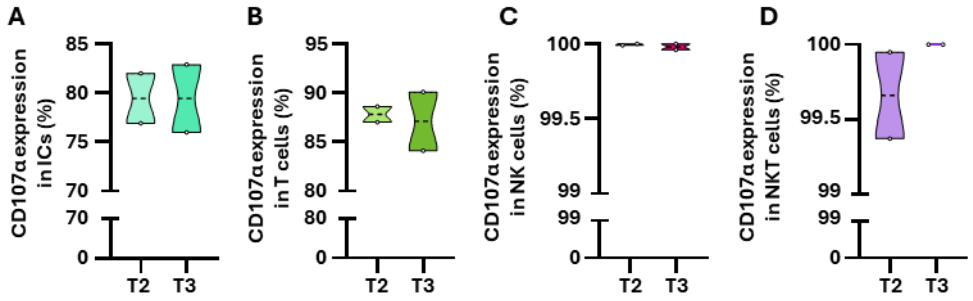

Figure R20. CD107α expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice. CD107α expression in the total pool of immune cells (A), T cells (B), NK cells (C) and NKT cells (D), as assessed by flow cytometry. Student's t-test vs T2.

When the analysis was performed separating CB_2R^+ and CB_2R^- cells, increased CD107α levels in T3 compared to T2 were detected in both populations when total immune cells were studied and this increase was more evident in CB_2R-expressing cells (**Figure R21A**).In this case, T cells may be responsible for these changes in CB_2R^+ cells as CD107α expression augmented with tumour size in this cell population. However, they did not contribute to the

observed changes in CD107α levels in CB_2R^- cells as no significant differences were detected between T2 and T3 (**Figure R21B**). In NK and NKT cells, the marker remained unchanged with tumour progression, regardless CB_2R expression (**Figures R21C and R21D**, respectively). These results contrast with our previous pooled analyses where CD107α expression did not change between T2 and T3. Nonetheless, they indicate that CB_2R does not play a significant role in regulating the cytotoxic capacity of immune cells within the BTME.

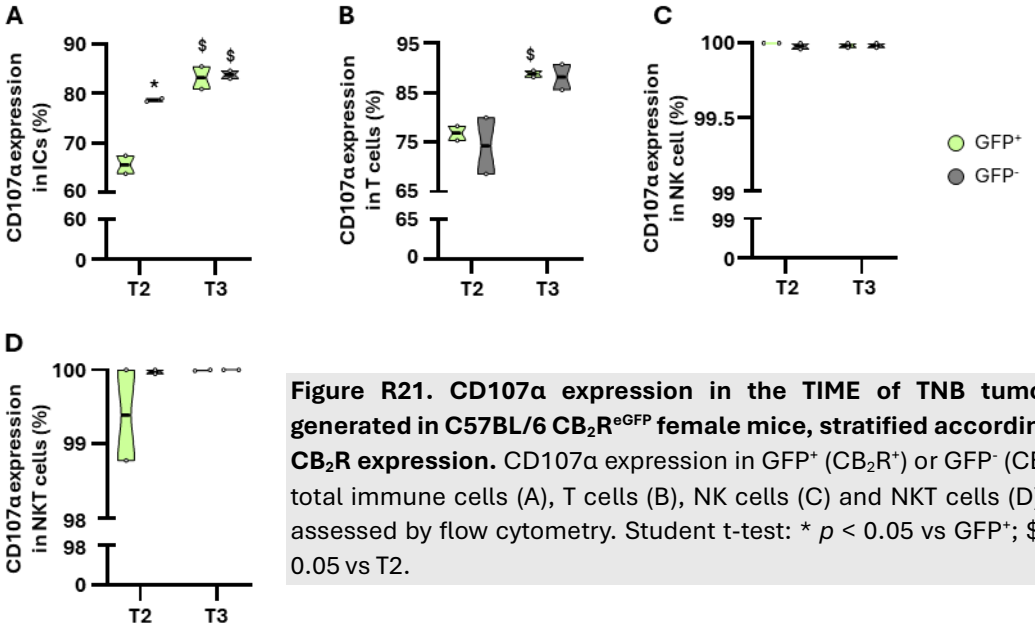

Figure R21. CD107α expression in the TIME of TNB tumours generated in C57BL/6 CB_2R^{eGFP} female mice, stratified according to CB_2R expression. CD107α expression in GFP^+ (CB_2R^+) or GFP^- (CB_2R^-) total immune cells (A), T cells (B), NK cells (C) and NKT cells (D), as assessed by flow cytometry. Student t-test: * $p < 0.05$ vs GFP^+; $ $p < 0.05$ vs T2.

Taken together, our results demonstrate that CB_2R is expressed by immune cells and that the BTME could modulate its levels to, we hypothesise, favour a less conductive microenvironment for effective antitumoural responses. Moreover, our data suggest that CB_2R plays a role in regulating the PD-1/PD-L1 axis at two distinct levels: i) by increasing the threshold required for cytotoxic T cell activation (i.e., limiting PD-1 expression in early tumour stages), and ii) by promoting immunosuppressive signalling mediated by PD-L1, particularly in myeloid cells. This novel insight provides a strong rationale for further investigation into the function of CB_2R in the BTIME, which will be explored in the following section of this thesis.

AIM 2. ANALYSIS OF THE ROLE OF BTME-CB_2R IN THE INITIATION AND PROGRESSION OF BC

It has been reported that CB_2R expressed within the TME can exert both pro- or antitumourigenic effects in murine models of melanoma, colon and lung cancer, among others (Gruber et al., 2021; Iden, Raphael-Mizrahi, Awida, et al., 2023; Sarsembayeva et al., 2023; X. Xiong et al., 2022). However, its role in BC remains poorly understood. Its general deletion (i.e., both in tumour and non-tumour cells) has been shown to delay tumour onset and to decrease tumour multiplicity and growth as well as lung metastasis formation in a mouse model of HER2-enriched metastatic BC (Pérez-Gómez et al., 2015). In this second objective, we sought to elucidate the specific role of TME-CB_2R (i.e., expressed in non-cancer cells) in BC initiation and progression, particularly focusing on the immune infiltrate. To this end, we used genetically engineered mouse models in which CB_2R expression had been knocked out.

IN SILICO ANALYSIS OF THE EXPRESSION OF CB_2R IN HUMAN BC SAMPLES

We first conducted transcriptomic analyses of publicly available datasets to investigate CB_2R expression within the breast stroma. Our results demonstrated that the cannabinoid receptor was significantly upregulated in the tumour-associated stroma compared to non-transformed stroma (**Figure R22A**). Additionally, we sought to determine whether there might be a relationship between elevated CB_2R expression and poorer patient prognosis. Analysis of the RNA-seq dataset published by Finak and collaborators (Finak et al., 2008) showed no statistically significant association between the risk of relapse regardless and BTME-CB_2R levels (**Figure R22B, left panel**). However, in the specific case of TNBC patients, BTME-CB_2R was significantly higher in individuals who experienced recurrence compared to those whose disease remained stable (**Figure R22B, right panel**).

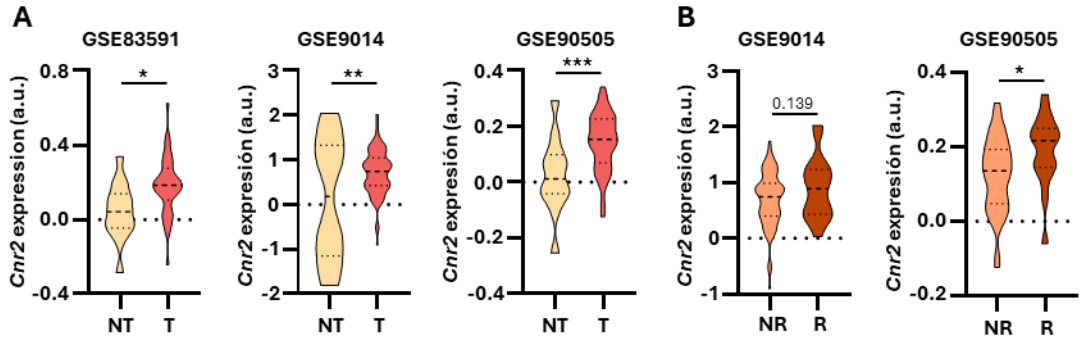

Figure R22. CB_2R mRNA expression in non-transformed and tumour-associated stroma from human BC samples. (A) CB_2R expression in non-transformed (NT) and tumoural (T) stroma. (B) CB_2R expression in tumoural stroma stratified according to patient recurrence. NR: no relapse. R: relapse. Data were obtained from the microarray datasets published by Liu et al., 2017 (accession number: GSE83591), Finak et al., 2008 (accession number: GSE9014) and Saleh et al., 2017 (accession number: GSE90505). Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NT or NR.

These findings suggest that BTME-CB_2R may contribute to tumour aggressiveness, particularly in TNBC.

ROLE OF TME-CB_2R IN DEFINING THE BASELINE COMPOSITION OF SPLENIC IMMUNE CELLS

Given our particular interest in immune cells, we began by characterising the potential influence of CB_2R in defining the baseline immune cell composition of the spleen. To this aim, we analysed the consequences of its complete inactivation on the immune status of non-tumour bearing (i.e., naive) female mice. For this purpose, we used CB_2R wild-type ($CB_2R^{+/+}$) and CB_2R KO mice ($CB_2R^{-/-}$) generated in two different genetic backgrounds: FVB/NJ and C57BL/6.

Flow cytometry analysis of the spleens of 8-10-week-old FVB/NJ MMTVneu females revealed no differences in immune cell frequencies, including T cell subpopulations, between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ animals (**Figure R23A**), suggesting that the mice had no alteration in global immune cells at steady state. However, in animals with a C57BL/6 background, cannabinoid receptor deletion did not change the percentage of total immune cells in the spleen but resulted in significant alterations in certain subsets (**Figure R23B**). Specifically, we observed a reduction in the percentages of B cells, accompanied by an increase in T, NK and NKT cell frequencies. Differences were also evident among T cell subsets: helper T cells (CD4) increased while cytotoxic T (CD8) cells diminished in $CB_2R^{-/-}$ animals. These results indicated a CB_2R-dependent disruption of the immune cell homeostasis in the C57BL/6 genetic background.

As highlighted in the previous section of this thesis, T cells—and more specifically cytotoxic T cells—are the immune cell population in which CB_2R expression undergoes the most significant changes between naive and activated states. To more specifically assess the contribution of CB_2R to T cell distribution, we generated the C57BL/6 CD4-Cre:CB_2R^{eGFP} mouse model (hereafter on CD4-$CB_2R^{-/-}$), in which cannabinoid receptor deletion was restricted to this population (see Materials and Methods). Comparative analysis of the spleens of these mice and their C57BL/6 CB_2R^{eGFP} controls showed that the absence of CB_2R in T cells decreased the proportion of cytotoxic T cells, without affecting other immune cell subsets (**Figure R23C**).

Taken together, these results suggest that CB_2R regulates the overall homeostasis of immune cell populations within the spleen of mice, but only in specific genetic backgrounds. Moreover, in C57BL/6 female mice, CB_2R expression specifically in T cells may be necessary to maintain the balance of cytotoxic T cells. Together, these observations imply that CB_2R may play a functionally relevant role in the physiopathology of the immune system and strengthen the rationale for investigating its contribution to the regulation of antitumour immunity.

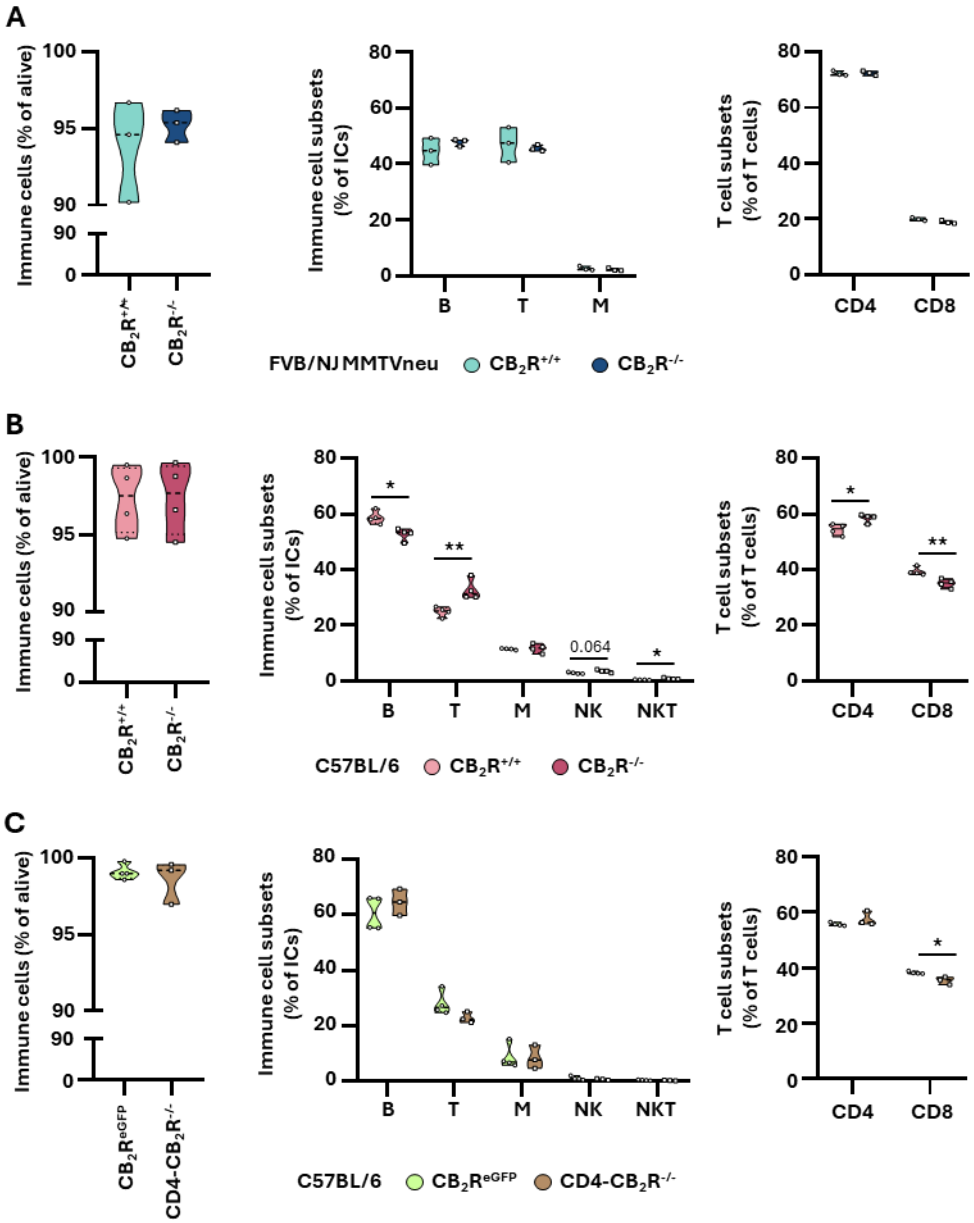

Figure R23. Splenic immune cell composition in non-tumour bearing female mice. Percentage of the indicated immune cell populations in spleens harvested from (A) FVB/NJ MMTVneu $CB_2R^{+/+}$ and $CB_2R^{-/-}$ (light and dark blue, respectively), (B) C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ (light and dark pink, respectively) and (C) C57BL/6 CB_2R^{eGFP} and CD4-$CB_2R^{-/-}$ (green and brown, respectively) animals. The left panels show the total percentage of total splenic immune cells, while the middle and right panels show the percentage of the main immune or T cell subsets, respectively. Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs $CB_2R^{+/+}$ (A and B) or CB_2R^{eGFP} (C).

CHARACTERISATION OF C57BL/6 $CB_2R^{+/+}$ AND $CB_2R^{-/-}$ SPLENOCYTES

To further investigate the role of CB_2R in antitumour immunity, we aimed to determine whether the differences observed in splenic immune composition in the C57BL/6 background could also translate into functional differences. To this aim, we characterised the basal and activated phenotypes of splenocytes isolated from non-tumour bearing females.

In the absence of any activation stimulus, we observed no significant differences between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ immune cells in the expression of immune surveillance markers, such as PD-1, CD69 or CD107α, either in the total pool of immune cells or specifically within T cells (**Figure R24A**). Similarly, we did not detect changes in the production of interferon-γ (IFN-γ), which was almost undetectable in the supernatant of unstimulated cultured splenocytes (**Figure R24B**).

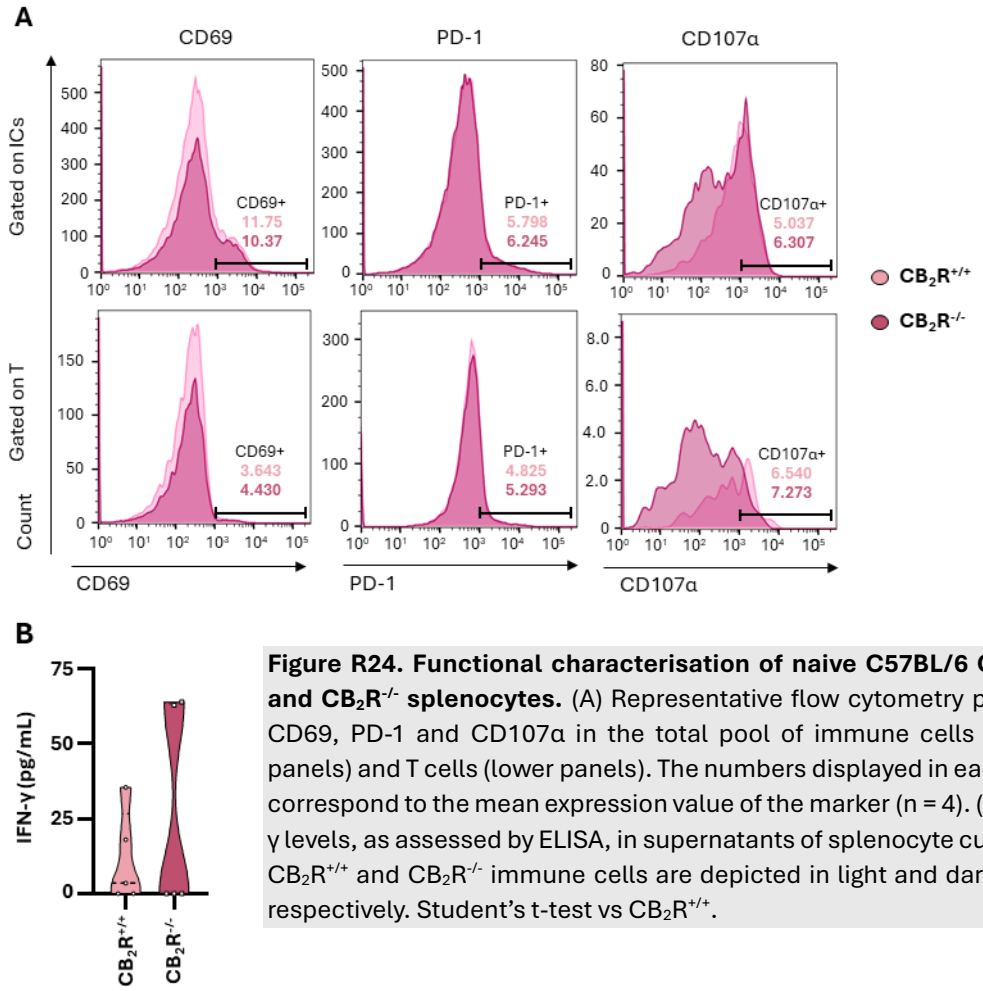

Figure R24. Functional characterisation of naive C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes. (A) Representative flow cytometry plots of CD69, PD-1 and CD107α in the total pool of immune cells (upper panels) and T cells (lower panels). The numbers displayed in each plot correspond to the mean expression value of the marker (n = 4). (B) IFN-γ levels, as assessed by ELISA, in supernatants of splenocyte cultures. $CB_2R^{+/+}$ and $CB_2R^{-/-}$ immune cells are depicted in light and dark pink, respectively. Student's t-test vs $CB_2R^{+/+}$.

Given the importance of the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling in regulating immune cell function (Owen et al., 2019) and the potential interactions identified by proteomic analysis between CB_2R and some key elements of this cascade (the IFN-γ receptor (IFNGR) and the phosphatase PTP1B) (Seijo et al., manuscript submitted), we also assessed the expression of these proteins by Western blot (**Figure R25**). No differences were detected in the total levels of IFNGR2, JAK1, STAT1, STAT3 or TC-PTP (a PTP1B-related small protein tyrosine phosphatase) between genotypes. However, $CB_2R^{-/-}$ cells displayed a significant reduction in the expression of IFNGR1 and PTP1B. While IFNGR1 downregulation may be a feedback mechanism to limit excessive inflammation, reduced PTP1B levels could instead result in a more sustained activation of the JAK/STAT pathway.

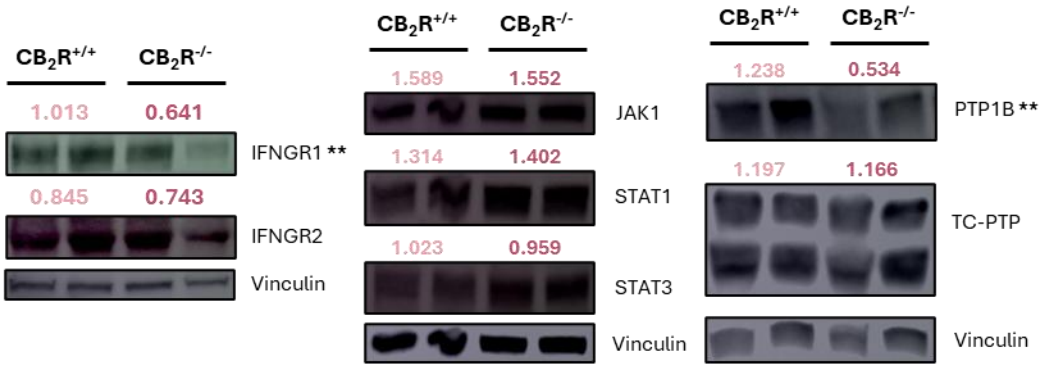

Figure R25. Analysis of the JAK/STAT signalling pathway in naive C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes. Representative Western blot analysis showing the IFN-γ receptor monomers (left), JAK/STAT pathway components (middle) and negative regulatory phosphatases (right). Densitometric values were normalised to Vinculin and are shown above each blot (n = 4). Student's t-test: ** $p < 0.01$ vs $CB_2R^{+/+}$.

Taken together, our results indicate that while CB_2R does not substantially influence the basal expression of immunosurveillance markers or the production of cytotoxic effector molecules in resting immune cells, it may contribute to raising the activation threshold for immune responses upon stimulation. Further experiments will be required to confirm this hypothesis.

Next, we aimed to investigate whether CB_2R modulates the activation capacity of splenocytes. To this end, splenocytes isolated from $CB_2R^{+/+}$ and $CB_2R^{-/-}$ animals were stimulated for 24h with the T cell mitogen Concanavalin A, hereafter referred to as ConA. No significant differences were found between genotypes in the expression of activation markers such as CD69 or PD-1, either in the total pool of immune cells or specifically within T cells. Similarly, overall expression of the degranulation marker CD107α was comparable; however, CB_2R-lacking T cells tended to express higher levels of CD107α, although this did not reach statistical significance (p = 0.0876) (**Figure R26A**). To further assess the functionality of these cells, we performed immunofluorescence assays to detect intracellular granzyme B (GrzB). These experiments revealed no significant differences in the accumulation of GrzB in CB_2R-expressing and CB_2R-lacking cells (**Figures R26B and R26C**). Consistently, IFN-γ release into the supernatant of ConA-activated splenocytes showed no differences between genotypes (**Figure R26D**).

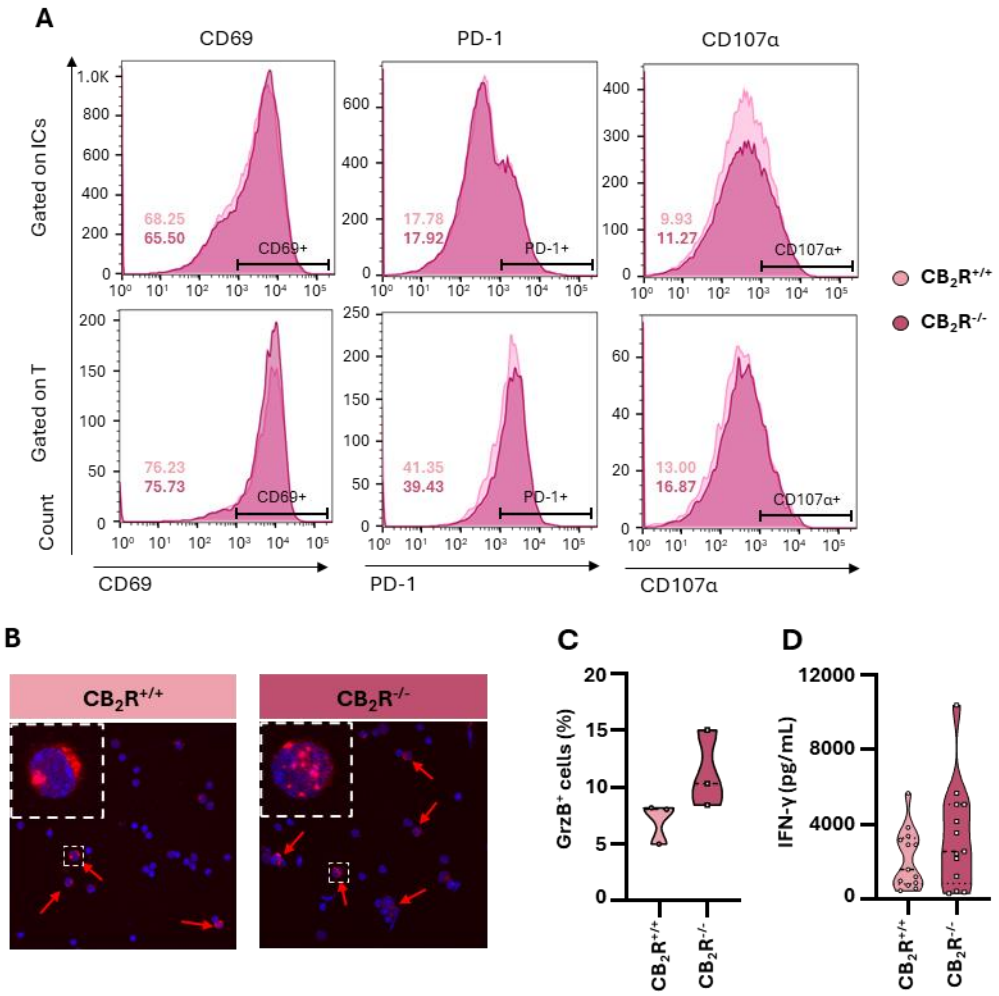

Figure R26. Functional characterisation of ConA-stimulated C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes. (A) Representative flow cytometry plots of CD69, PD-1 and CD107α in the total pool of immune cells (upper panels) and T cells (lower panels). Numbers displayed in each plot correspond to the mean expression value of the marker (n = 4). (B) Representative immunofluorescence of GrzB in ConA-activated splenocytes. $GrzB^+$ cells are pointed with red arrows. Scale bar 40 µm. (C) Quantification of $GrzB^+$ cells from panel B. (D) IFN-γ levels, as assessed by ELISA, in supernatants of ConA-stimulated splenocyte cultures. $CB_2R^{+/+}$ and $CB_2R^{-/-}$ immune cells are depicted in light and dark pink, respectively. Student's t-test vs $CB_2R^{+/+}$.

Together, although none of the differences in between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ genotypes reached statistical significance, our observations point to a role of CB_2R in restraining T cell cytotoxic activity. Nonetheless, further experiments will be required to clarify the function of CB_2R on cytotoxic granule activity.

To further assess this possibility, we analysed the killing capacity of CB_2R-expressing and CB_2R-lacking splenocytes in co-cultures with cancer cells. When the analysis was performed in the C57BL/6 model, we observed that the killing capacity tended to be higher in $CB_2R^{-/-}$ splenocytes than in their CB_2R-positive counterparts across the different effector-to-target (E:T) ratios tested, although these differences did not reach statistical significance (**Figure R27A**).

A similar trend was observed in co-cultures with splenocytes isolated from the CB_2R^{eGFP} model: a (not significant) increased in the cytotoxic capacity of CD4-$CB_2R^{-/-}$ cells vs that of CB_2R-positive splenocytes (**Figure R27B**).

These results support the notion that CB_2R may attenuate the cytotoxicity not only of T cells, but also of other immune cell populations.

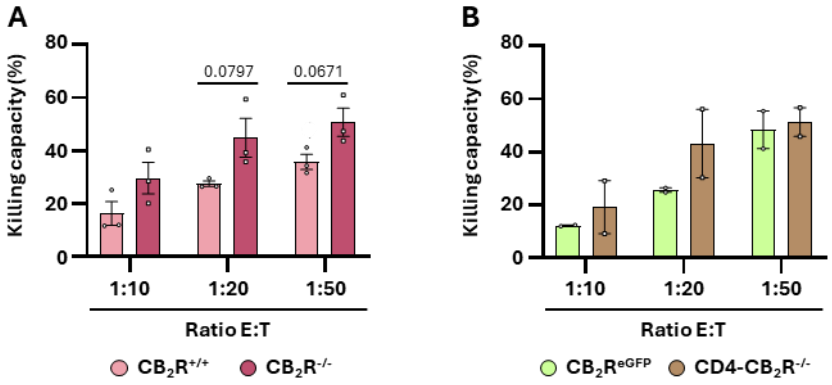

Figure R27. Analysis of the killing capacity of splenocytes. (A-B) Killing capacity of C57BL/6 $CB_2R^{+/+}$ (light pink bars) and $CB_2R^{-/-}$ (dark pink bars) splenocytes (A) or CB_2R^{eGFP} (green bars) and CD4-$CB_2R^{-/-}$ (brown bars) splenocytes (B) in co-cultures with AT3 cancer cells, calculated as 100 minus the percentage of viable AT3 cells in co-culture, previously normalised to the viability of AT3 cells cultured alone. Student's t-test vs $CB_2R^{+/+}$ (A) or CB_2R^{eGFP} (B).

Taken together, these results suggest that CB_2R may limit immune cell activation and reduce their effector capacity, which could translate into impaired immunosurveillance, thereby facilitating tumour progression. Nonetheless, because immune cell functionality can be further shaped by the TME, potentially amplifying or counteracting these intrinsic differences, it is crucial to extend these analyses to tumour-infiltrating lymphocytes to further corroborate our hypothesis.

ROLE OF TME-CB_2R IN THE INITIATION AND PROGRESSION OF BC

Next, we studied the functional role of CB_2R expressed by immune cells in BC progression. To this aim, we analysed the consequences of CB_2R deletion in the TME in terms of tumour progression and immune infiltration across different syngeneic mouse models of BC. Specifically, tumours were generated by orthotopic injection of syngeneic BC cell lines into the previously described mice and harvested at different time points (**Figure R28**).

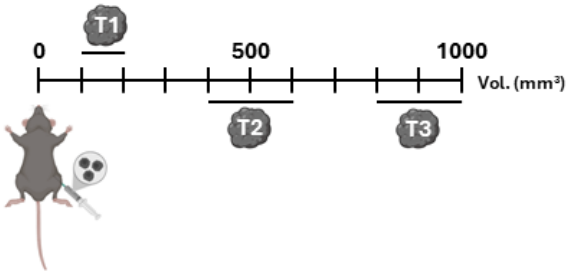

Figure R28. Experimental set up for the analysis of the functional relevance of CB_2R in the BTIME. Syngeneic BC cell lines were orthotopically implanted in immunocompetent female mice. Tumours were harvested at three different timepoints, defined according to tumour volume (Vol.): 100-200 (T1), 400-600 (T2) and 800-1000 (T3) mm^3.

ROLE OF TME-CB_2R IN THE INITIATION AND PROGRESSION OF LUMINAL B TUMOURS

Although luminal BC is generally associated with a favourable prognosis, not all patients evolve or respond equally to treatment, underscoring the need to explore novel mechanisms involved in cancer development. To study whether TME-CB_2R may contribute to the progression of this disease, we generated luminal B tumours in C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ female mice by orthotopic injection of EO771 BC cells.

Monitoring of tumour growth in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females revealed no significant differences between in either tumour onset or growth rate, suggesting that CB_2R does not substantially impact the initiation or progression of luminal B breast tumours (**Figure R29**).

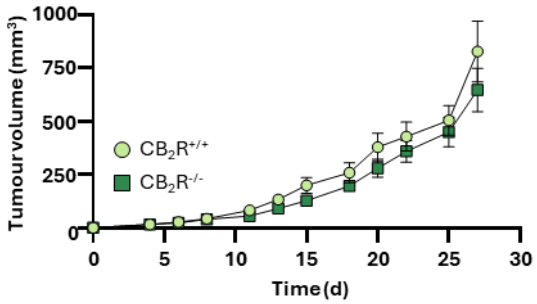

Figure R29. Tumour growth in a syngeneic luminal B BC mouse model. Tumour growth in C57BL/6 $CB_2R^{+/+}$ (light green circles) and $CB_2R^{-/-}$ (dark green squares) females orthotopically injected with luminal B EO771 cells. Two-way ANOVA.

Similarly, we did not find differences in immune cell infiltration at T3 between the two genotypes, neither in the total pool of tumour-infiltrating immune cells, nor in the major immune cell subsets nor within the principal T cell subpopulations (**Figure R30**). As no

changes were detected in the percentages of helper (CD4) and cytotoxic (CD8) T cells, the CD4/CD8 ratios also remained similar between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumours (0.126 ± 0.025 y 0.128 ± 0.017, respectively).

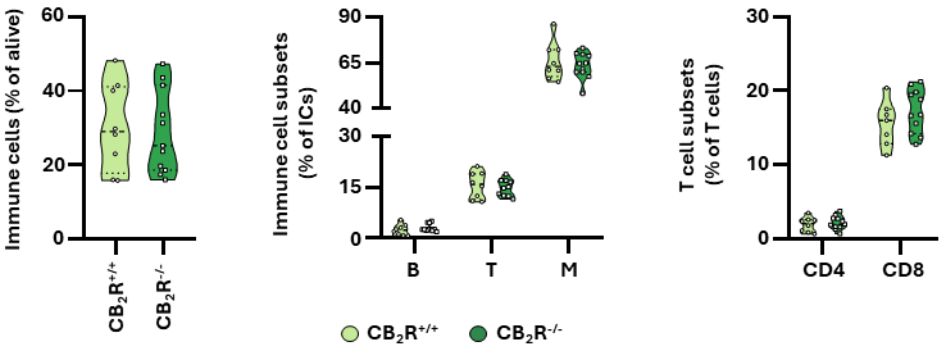

Figure R30. Immune composition of the TME in a syngeneic luminal B BC mouse model. Percentages of total tumour-infiltrating immune cells (left panel), major immune cell subsets (middle panel) and main T cell subpopulations (right panel) in tumours harvested at T3, as assessed by flow cytometry. C57BL/6 $CB_2R^{+/+}$ tumours are depicted in light green, while $CB_2R^{-/-}$ tumours are in dark green. Student's t-test vs $CB_2R^{+/+}$.

In the TME the role of soluble factors is critical to tipping the balance towards pro- or antitumoural responses (De Visser & Joyce, 2023). To address whether the deletion of CB_2R could also influence this component, we analysed the expression of various cytokines and chemokines using an antibody array (**Figure R31**).

Only of one soluble factor differed statistically between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumours: the complement component C5a, which was increased in the absence of CB_2R. Notably, C5a has differential effects in BC (pro-tumourigenic or antitumoural) depending on its concentration (Darling et al., 2015).

Figure R31. Analysis of the soluble component of the TME in EO771 tumours generated in C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females. (A) Representative image of the antibody array membranes showing the significantly and differentially expressed protein. (B) Densitometric quantification of C5a expression. Student's t-test: *** $p < 0.001$ vs $CB_2R^{+/+}$.

Taken together, these results indicate that TME-CB_2R may not have a significant impact in luminal B breast tumours, as its deletions does not result in notable changes in tumour

onset and growth or in the composition of the BTME (neither in terms of immune infiltration nor in the non-cellular compartment).

ROLE OF TME-CB_2R IN THE INITIATION AND PROGRESSION OF HER2-ENRICHED TUMOURS

Despite advances achieved in HER2-targeted therapies, a considerable percentage of HER2-enriched BC patients continue to experience treatment resistance and early relapse, highlighting the need to identify novel mechanisms driving tumour progression and therapy resistance. Previous work from our group showed that CB_2R expressed by cancer cells promotes generation and progression of HER2-enriched breast tumours (Pérez-Gómez et al., 2015). In that study, among other tools, a mouse model of HER2-enriched metastatic BC was used: the FVB/NJ MMTVneu mouse, in which the proto-oncogene neu (the rat orthologue of HER2) is under the control of a mammary-specific promoter (murine mammary tumour virus, MMTV), resulting in the spontaneous generation of HER2-positive metastatic breast tumours. Here we used two different models based on the FVB/NJ MMTVneu mouse to assess the potential contribution of TME-CB_2R to HER2-positive tumour initiation and progression (**Figure R32**). The first model was the original FVB/NJ MMTVneu mouse, in which tumours were allowed to develop spontaneously. In this model, CB_2R was absent in all cells (both non-tumour and tumour) in the $CB_2R^{-/-}$ genotype. In the second model, syngeneic N202-1A tumour cells were orthotopically injected into young $CB_2R^{+/+}$ and $CB_2R^{-/-}$ FVB/NJ MMTVneu mice (8-10-week-old), at an age when the neu transgene is assumed to be inactive and, therefore, the process of spontaneous tumour development has not yet commenced (Caffarel et al., 2010). Since the N202-1A cell line expresses CB_2R, this model allows studying the effect of CB_2R knockout specifically in the host's organism.

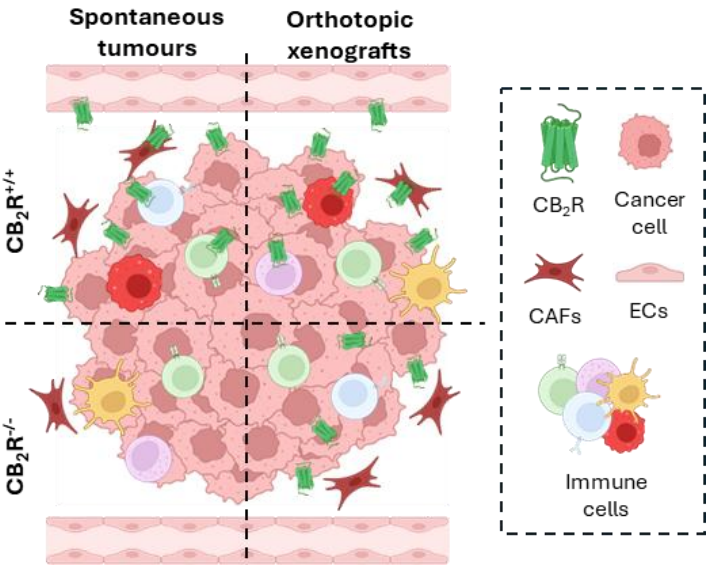

Figure R32. Animal models of HER2-enriched BC. Schematic representation of the two MMTVneu-based models of HER2-enriched BC used in this section. The model on the left depicts the original MMTVneu mouse, in which tumours develop spontaneously. In this case $CB_2R^{-/-}$ animals lack CB_2R in both tumour and non-tumour cells. The model on the right represents young MMTVneu animals

orthotopically injected with the syngeneic BC cell line N202-1A. In this case, $CB_2R^{-/-}$ animals lack the receptor in all their organism (including the TME), while tumour cells retain CB_2R expression.

First, we analysed tumour growth in the original FVB/NJ MMTVneu model and found no significant differences in tumour volume when comparing age-matched $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females (**Figure R33A**). We then assessed the immune composition of tumours, regardless of tumour size, by flow cytometry. Overall, the absence of CB_2R led to a trend toward increased infiltration of total immune cells (**Figure R33B, left panel**). Upon further analysis, we discovered a significant increase in B and T cell percentages—typically associated with better patient prognosis (S. Adams et al., 2015; Kuroda et al., 2021; Kurozumi et al., 2019; Stanton & Disis, 2016)—in $CB_2R^{-/-}$ females, without changes in the frequencies of myeloid cells (**Figure R33B, middle panel**). Regarding T cell subpopulations, CB_2R deletion resulted in an increase proportion of both helper (CD4) and cytotoxic (CD8) T cells, although it only reached statistical significance in the former (**Figure R33B, right panel**). These changes were accompanied by a lower CD4/CD8 ratio (0.875 ± 0.1105 and 0.610 ± 0.0795 in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumours, respectively) in CB_2R-lacking tumours, a parameter that has been reported to correlate with favourable overall survival (OS) and relapse-free survival (Huang et al., 2015). The observed difference nearly reached statistical significance ($p = 0.072$).

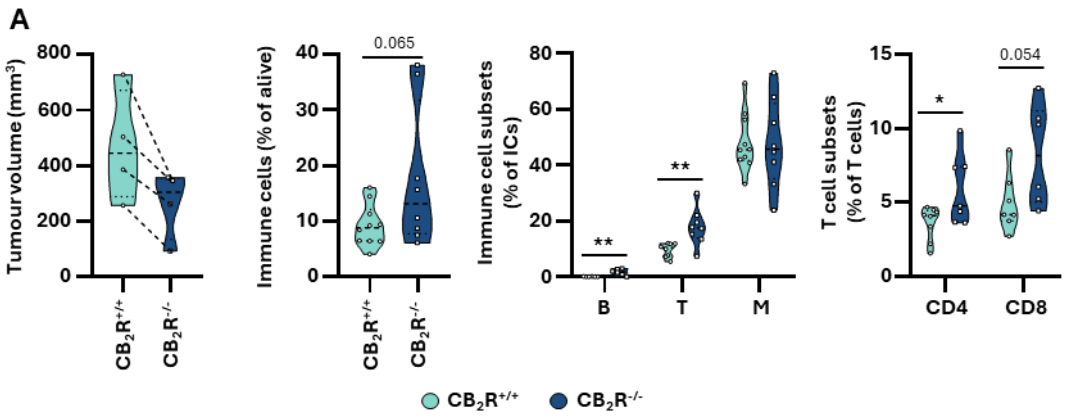

Figure R33. Immune composition of the TME in the MMTVneu spontaneous model of HER2-enriched BC. (A) Tumour volume in FVB/NJ MMTVneu $CB_2R^{+/+}$ and $CB_2R^{-/-}$ female mice (40-50-week-old), with lines connecting tumours from age-matched individual. (B) Percentages of total immune infiltrating cells (left panel), major immune cell subsets (middle panel) and main T cell subpopulations (right panel), as assessed by flow cytometry. FVB/NJ MMTVneu $CB_2R^{+/+}$ tumours are depicted in light blue, while $CB_2R^{-/-}$ tumours are in dark blue. Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs $CB_2R^{+/+}$.

Next, we studied potential changes in the non-cellular component of the TME upon CB_2R deletion by analysing the expression of 40 soluble factors using an antibody array (**Figure R34**). We only observed two proteins that were significantly upregulated in $CB_2R^{-/-}$ tumours: the cytokine IL-16, which has been reported to have both tumour-suppressive (Wen et al., 2025) and pro-metastatic (Donati et al., 2017) functions in BC depending on the context, and the metalloproteinase inhibitor TIMP-1, which also has a dual role as both pro-oncogenic and tumour suppressor (Justo & Jasiulionis, 2021).

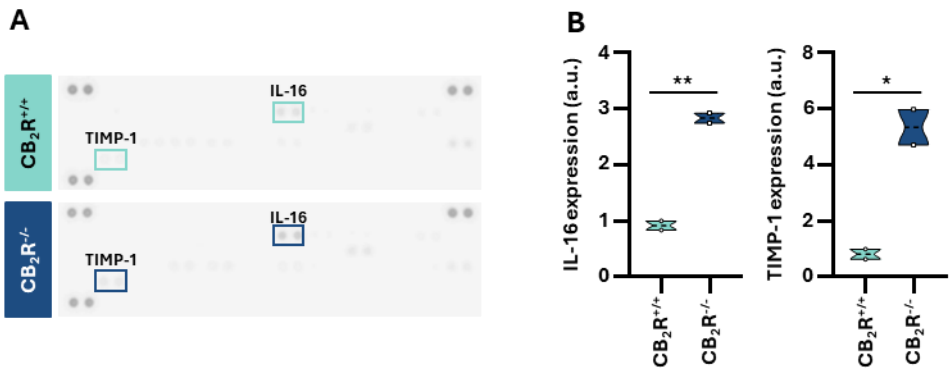

Figure R34. Analysis of the soluble component of the TME in the spontaneous FVB/NJ MMTVneu mouse model of HER2-enriched BC. (A) Representative image of the antibody array membranes showing the significantly and differentially expressed proteins. (B) Densitometric quantification of the proteins marked in A. Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs $CB_2R^{+/+}$.

Altogether, the increased tumour growth previously reported by our group (Pérez-Gómez et al., 2015) and the lower immune infiltration associated with better BC outcomes support an oncogenic role of CB_2R in HER2-enriched tumours. Furthermore, the pleiotropic effects of the differentially expressed soluble factors between CB_2R-expressing and CB_2R-lacking tumours underscore the need for further investigation into the specific role of CB_2R in this tumour subtype.

However, because this first model involves CB_2R deletion in both tumour and non-tumour cells, it does not allow us to delineate the specific contribution of TME-CB_2R to the observed effects. To address this, we orthotopically injected the syngeneic HER2-enriched BC cell line N202-1A in 8–10-week-old FVB/NJ MMTVneu $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females.

Tumours were first detected at 15 days post-implantation, although significant growth did not occur until after one month. We did not observe differences in tumour onset between the two genotypes. However, as in the pure FVB/NJ MMTVneu model, tumour growth tended to be slightly higher in $CB_2R^{+/+}$ animals (**Figure R35**), suggesting a subtle pro-tumoural role of the stromal CB_2R.

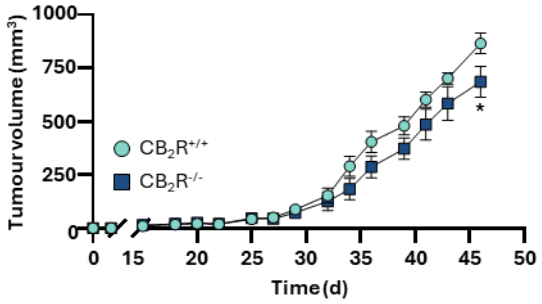

Figure R35. Tumour growth in the orthotopic xenograft model of HER2-enriched BC. Volume of N202-1A-derived tumours generated in FVB/NJ MMTVneu $CB_2R^{+/+}$ (light blue circles) and $CB_2R^{-/-}$ (dark blue squares) females. Two-way ANOVA: * $p < 0.05$ vs $CB_2R^{+/+}$.

No significant differences were found in the total immune infiltration between the two genotypes in tumours harvested at T3 (**Figure R36, left panel**). Similarly, the percentages of myeloid and T cells, the two major infiltrating populations, as well as B cells, did not differ between groups (**Figure R36, middle panel**). In contrast, the proportion of innate immune

NK cells was significantly increased in $CB_2R^{-/-}$ tumours (**Figure R36, middle panel**), a feature that has been generally associated with improved outcomes in BC patients (Nersesian et al., 2021). Regarding T cell subpopulations, we did not detect changes in their percentages (**Figure R36, right panel**), and the CD4/CD8 ratio was very similar between genotypes (1.070 ± 0.259 and 1.039 ± 0.194 for $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumours, respectively).

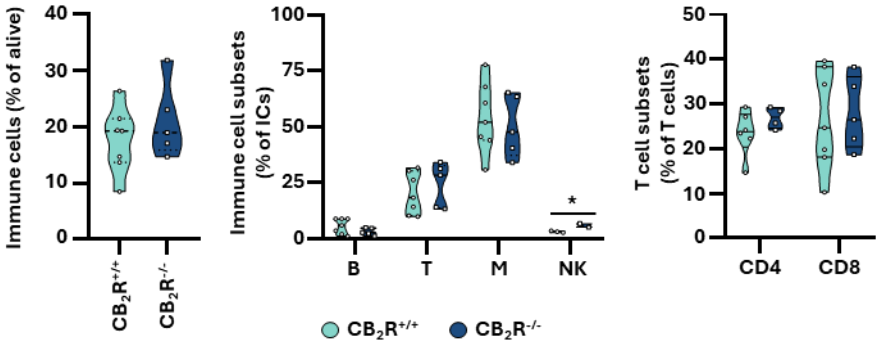

Figure R36. Immune composition of the TME in N202-1A orthotopic tumours generated in FVB/NJ MMTVneu mice. Percentages of total tumour-infiltrating immune cells (left panel), major immune cell subsets (middle panel) and main T cell subpopulations (right panel) in tumours harvested at T3. FVB/NJ MMTVneu $CB_2R^{+/+}$ tumours are shown in light blue, while $CB_2R^{-/-}$ tumours are in dark blue. Student's t-test: * $p < 0.05$ vs $CB_2R^{+/+}$.

These results reveal only subtle changes in tumour growth and tumour infiltration upon CB_2R deletion in the TME, which suggests that this receptor (expressed in this particular compartment) does not play a major role in modulating HER2-enriched breast tumour initiation and progression. However, when considered together with the results obtained in the original FVB/NJ MMTVneu model (where CB_2R was also knocked down in cancer cells), they support the notion that CB_2R expressed by tumour cells has a potent pro-oncogenic role that is not only attributable to its role in driving HER2-pro-tumourigenic signalling, as previously reported by our group (Pérez-Gómez et al., 2015), but also to its capacity to limit the infiltration of immune cells generally associated with improved outcomes in BC patients.

ROLE OF TME-CB_2R IN THE INITIATION AND PROGRESSION OF TNB TUMOURS

TNBC is the BC subtype with the poorest prognosis due to its aggressive behaviour and the lack of specific therapeutic targets and therefore selective treatments. However, it is considered the most immunogenic subtype, making it the best candidate for immunotherapy-based treatments. To investigate whether TME-CB_2R, given its high expression in immune cells, could contribute to the progression of this disease, we performed *in vivo* experiments using a mouse model of this pathology. Specifically, TNB tumours were generated in C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ female mice by orthotopic injection of AT3 BC cells.

Although we did not observe differences in tumour onset between the two genotypes, the growth rate of $CB_2R^{-/-}$ tumours was significantly slower than that of their $CB_2R^{+/+}$ counterparts, which reached the T3 endpoint within 30 days after BC cell injection (**Figure R37**). These growth kinetics suggest that stromal CB_2R may play a critical role in promoting tumour growth, particularly in TNBC.

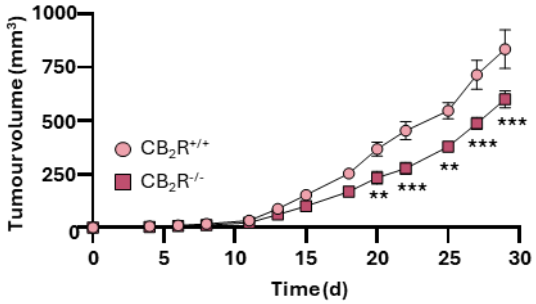

Figure R37. Tumour growth in a syngeneic TNBC mouse model. Growth of AT3-derived tumours generated in C57BL/6 $CB_2R^{+/+}$ (light pink circles) and $CB_2R^{-/-}$ (dark pink squares) females. Two-way ANOVA: ** $p < 0.01$, *** $p < 0.001$ vs $CB_2R^{+/+}$.

Flow cytometry analysis of the BTIME revealed distinct infiltration dynamics between genotypes. In $CB_2R^{+/+}$ animals, the percentage of total immune cells within tumours was relatively high (~ 35 %), remained largely unchanged from T1 to T2, and significantly diminished in large tumours (T3). Conversely, their $CB_2R^{-/-}$ littermates showed significantly lower infiltration in small tumours (~ 14 %), which increased at T2 and remained high at T3, ultimately reaching levels comparable to those of their $CB_2R^{+/+}$ counterparts (**Figure R38**).

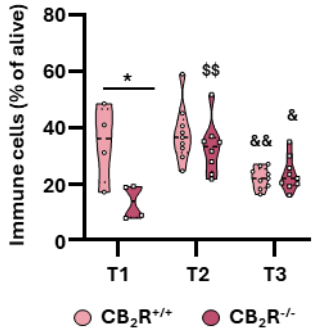

Figure R38. Immune composition of the TME in a syngeneic TNBC mouse model. Percentages of total tumour-infiltrating immune cells in tumours generated by orthotopic injection of AT3 cells in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) mice, and harvested at T1, T2 and T3. Student's t-test: * $p < 0.05$ vs $CB_2R^{+/+}$. One-way ANOVA: $$ $p < 0.01$ vs T1; & $p < 0.05$, && $p < 0.01$ vs T2.

The proportion of innate immune NK cells within the tumours tended to be higher in animals lacking CB_2R at T1. This percentage changed as tumours grew following a bell-shape behaviour (it significantly rose at T2 and nearly disappeared by T3) that similar in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ mice (**Figure R39A**). NKT cells represented a relatively small proportion of the BTIME, without significant differences between CB_2R-expressing and CB_2R-lacking tumours, and their frequency significantly decreased upon tumour growth in both genotypes (**Figure R39B**). Concerning myeloid cells, their percentages in the tumours was higher in $CB_2R^{-/-}$ animals at T1. While their proportion changed following a bell-shaped curve in $CB_2R^{+/+}$ animals (significantly increased at T2 and decrease to nearly initial levels at T3), myeloid infiltration in CB_2R-lacking tumours remained stable in medium tumours and decreased to levels similar to those of their CB_2R-expressing counterparts by T3 (**Figure R39C**). At early stages (T1), B cells were very abundant in tumours from $CB_2R^{+/+}$ animals, but virtually absent in those from animals lacking CB_2R. Its proportion significantly decrease as tumour grew in

$CB_2R^{+/+}$ mice, whereas significantly increased upon tumour growth in $CB_2R^{-/-}$ animals. (**Figure R39D**).

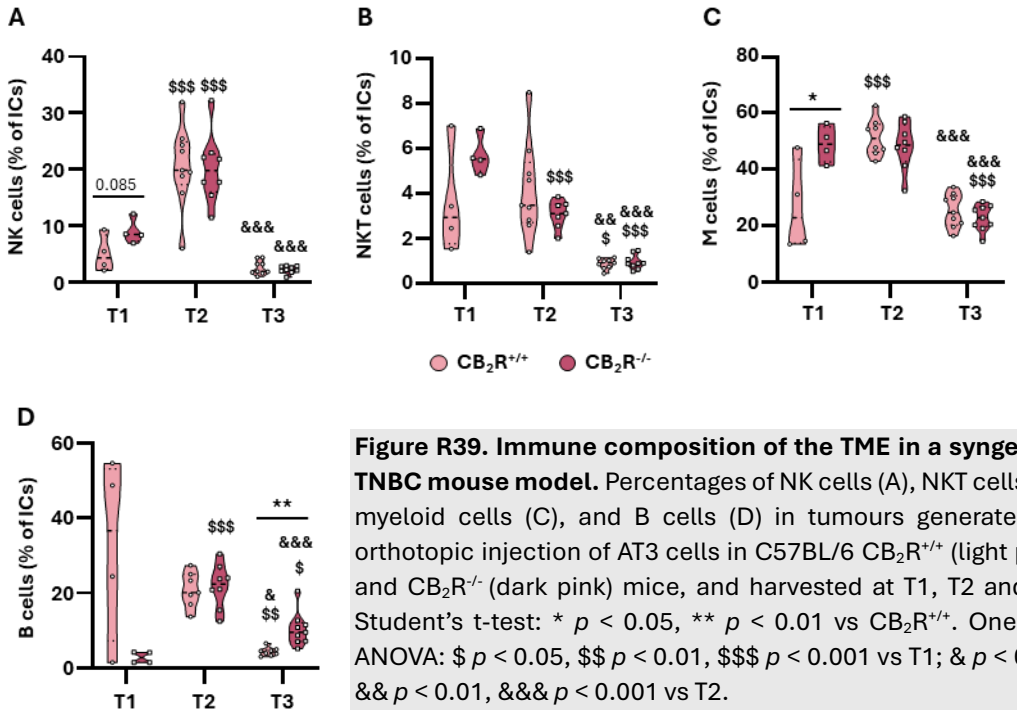

Figure R39. Immune composition of the TME in a syngeneic TNBC mouse model. Percentages of NK cells (A), NKT cells (B), myeloid cells (C), and B cells (D) in tumours generated by orthotopic injection of AT3 cells in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) mice, and harvested at T1, T2 and T3. Student's t-test: * $p < 0.05$, ** $p < 0.01$ vs $CB_2R^{+/+}$. One-way ANOVA: \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs T1; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ vs T2.

T cell percentage where very similar in both genotypes at T1 and followed an analogous dynamic, curve-shaped pattern as tumours grew, decreasing at T2 and recovering to nearly initial levels by T3, where their infiltration was significantly higher in $CB_2R^{-/-}$ tumours compared to $CB_2R^{+/+}$ ones (**Figure R40A**). Helper T cell proportion was virtually identical in both genotypes at T1 and remained mostly unchanged in $CB_2R^{+/+}$ mice until T3, where a small decrease was observed. The $CB_2R^{-/-}$ counterparts showed a similar behaviour, with a higher decrease in CD4 infiltration in T3 than in their CB_2R-expressing littermates (**Figure R40B**). The percentage of cytotoxic T cells was also similar in both genotypes at T1 and followed a comparable change pattern as tumours grew: it significantly decreases to nearly undetectable levels in T2 and recovered, and even exceeded, initial levels by T3. In CB_2R-lacking females, the CD8 T cell percentage ultimately became significantly higher in large tumours compared to their $CB_2R^{+/+}$ counterparts (**Figure R40C**). Finally, the dynamics of the T cell subpopulations resulted in similar CD4/CD8 ratios in T1 and T2, but a significantly lower ratio (usually associated with better prognosis) in large tumours in the absence of CB_2R (**Figure R40D**).

Taken together, these findings suggest that TME-CB_2R promotes an environment with reduced infiltration of innate immune cells displaying predominantly antitumoural functions—including NK and myeloid populations (Khan et al., 2023; Nersesian et al., 2021)—during the initial phases of tumourigenesis. Moreover, in advanced tumours, CB_2R appears to limit the proportions of adaptive immune cells, such as B and CD8 T cells, linked to better outcomes in BC patients (Kuroda et al., 2021; Oshi, Asaoka, et al., 2020).

In summary, these results indicate that the CB_2R expressed by the tumour stroma plays a pro-tumoural role in TNBC as its presence contributes to a BTIME composition that is less favourable for effective antitumour immune responses.

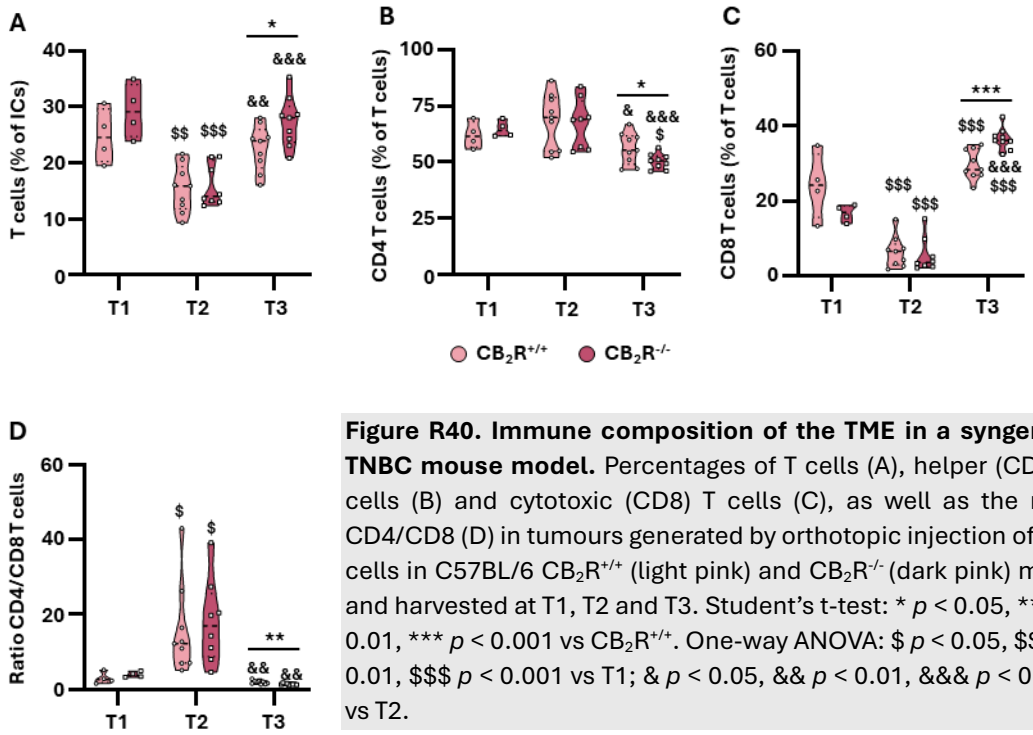

Figure R40. Immune composition of the TME in a syngeneic TNBC mouse model. Percentages of T cells (A), helper (CD4) T cells (B) and cytotoxic (CD8) T cells (C), as well as the ratio CD4/CD8 (D) in tumours generated by orthotopic injection of AT3 cells in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) mice, and harvested at T1, T2 and T3. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs $CB_2R^{+/+}$. One-way ANOVA: \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs T1; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ vs T2.

To further assess the role of TME-CB_2R, we evaluated the soluble component of the microenvironment at T3 using an antibody array (**Figure R41A**). We did not observe significant differences in any of the analysed molecules. However, two of them almost reached statistical significance: ICAM-1, which is implicated in metastasis (Taftaf et al., 2021) and immune evasion (W. Zhang et al., 2022), as well as lymphocyte-associated immunological activation (Q. Zhou et al., 2023); and CXCL12, a chemokine involved in BC cell survival and proliferation, angiogenesis and metastasis (Williams et al., 2010). Both factors were found to be downregulated in $CB_2R^{-/-}$ tumours compared to their $CB_2R^{+/+}$ counterparts (**Figure R41B**).

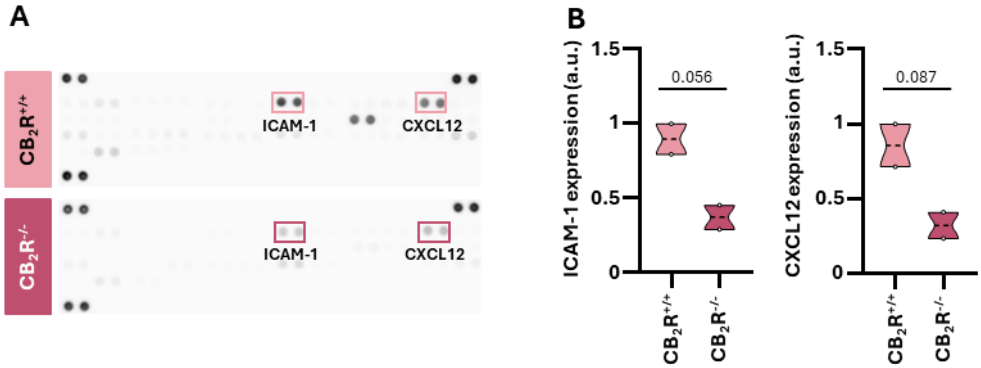

Figure R41. Analysis of the soluble component of the TME in AT3 tumours generated in C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females. (A) Representative image of the antibody array membranes showing the differentially expressed proteins (although the differences did not reach statistical significance). (B) Densitometric quantification of the proteins marked in A. Student's t-test vs $CB_2R^{+/+}$.

These results are in line with our previous observations pointing to a pro-oncogenic function of the CB_2R expressed in the TME, promoting an immunosuppressive microenvironment that favours tumour progression.

To continue investigating the contribution of TME-CB_2R to TNB tumour progression, we assessed the effect of CB_2R deletion on the expression of critical immune surveillance markers: immune checkpoint proteins (PD-1 and PD-L1), immune activation (CD69) and cytotoxic effector cell activity (CD107α).

PD-1 levels tended to be higher at the initial stages (T1) in $CB_2R^{-/-}$ immune cells. As tumours grew, an increase at T2 followed by a decrease to near initial values at T3 were observed in both genotypes, with no significant differences between them (**Figure R42A**). A comparable expression pattern was found when specifically analysing cytotoxic T cells across both genotypes: higher, although not statistically significant, PD-1 levels in T1 in CD8 T cells lacking CB_2R, and a similar bell-shaped behaviour in both genotypes as tumours grew (an increase at T2 followed by a decline at T3) (**Figure R42B**). Additionally, we evaluated the PD-1 expression ratio between CD8 and CD4 T cells, as it has been shown to positively correlate with progression-free survival and OS (Duchemann et al., 2022). Our results revealed a significant increase in this ratio at T3 compared to T1 and T2, with CB_2R-negative T cells displaying a significantly higher ratio than their CB_2R-expressing counterparts at this particular stage (**Figure R42C**).

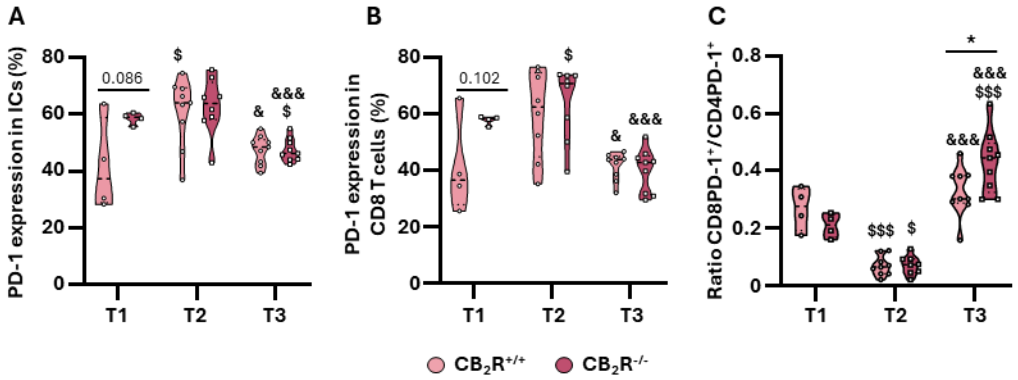

Figure R42. PD-1 expression in the TIME of TNB tumours. PD-1 expression in total immune cells (A) and CD8 T cells (B) in tumours generated in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) animals by orthotopic injection of AT3 cells, and harvested at T1, T2 and T3. (C) PD-1 expressing ratio on CD8 and CD4 lymphocytes. Student's t-test: * $p < 0.05$ vs $CB_2R^{+/+}$. One-way ANOVA: \$ $p < 0.05$, \$\$\$ $p < 0.001$ vs T1; & $p < 0.05$, &&& $p < 0.001$ vs T2.

These results suggest that CB_2R increases the activation threshold of cytotoxic T cells during the initial stages of tumour development. As the tumour progresses, the immunosuppressive TME appears to override the possible influence of CB_2R signalling, leading to a convergence of PD-1 expression levels regardless of CB_2R status.

PD-L1 expression within the total pool of immune cells was significantly higher in $CB_2R^{-/-}$ than in $CB_2R^{+/+}$ cells at T1. With tumour growth, PD-L1 levels showed a marked increase at

T2, followed by a decline below initial values at T3 in both genotypes (**Figure R43A**). When specifically analysing PD-L1 expression in helper T cells, higher levels were also observed in CB_2R-lacking CD4 T cells in small tumours (T1). PD-L1 expression progressively declined in both genotypes to nearly undetectable levels as tumour grew (**Figure R43B**). Changes in PD-L1 levels in myeloid cells could be responsible for the expression pattern observed when analysing total immune cells, as tumour progression was associated with increased PD-L1 expression at T2, followed by a pronounced decrease at T3 in both genotypes. However, no differences in its levels between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ myeloid cells were found at any tumour size analysed (**Figure R43C**), suggesting that CB_2R signalling does not to have significant influence on PD-L1 expression in this immune subset.

These results suggest that CB_2R may limit the activation of helper T cells at the initial phases of the antitumoural immune response, or alternatively, may prevent immunosuppression in this context. Further analyses will be needed to determine the exact role of CB_2R in this T cell subset.

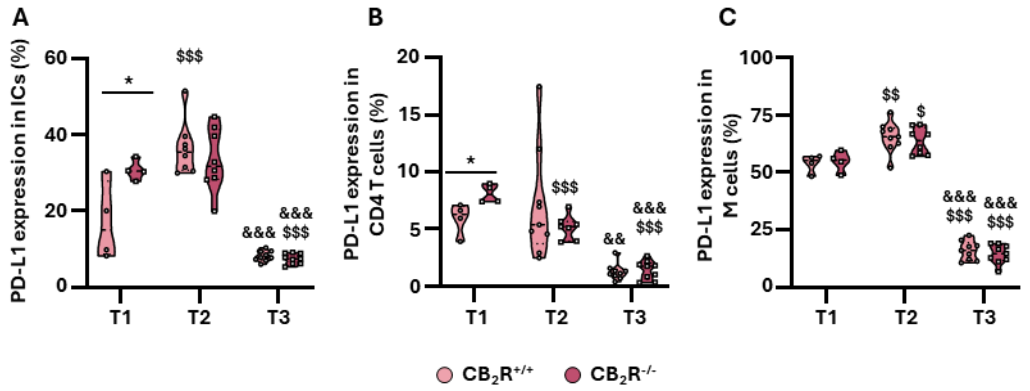

Figure R43. PD-L1 expression in the TIME of TNB tumours. PD-L1 expression in total immune cells (A), helper T cells (B), and myeloid cells (C) in tumours generated in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) animals by orthotopic injection of AT3 cells, and harvested at T1, T2 and T3. Student's t-test: * $p < 0.05$ vs $CB_2R^{+/+}$. One-way ANOVA: \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs T1; &&& $p < 0.001$ vs T2.

Taken together, the expression pattern of the PD-1/PD-L1 axis may suggest that CB_2R diminished the efficacy of the antitumoural immune response in the earlier stages of carcinogenesis. The increased expression of PD-1 in CB_2R-lacking CD8 T cells and PD-L1 in $CB_2R^{-/-}$ CD4 T cells compared to their CB_2R-expressing counterparts could reflect an intense immune activation and a feedback mechanism to prevent potential damage associated with such hyperactivation.

The analysis of the activation marker CD69 showed a progressive decrease in its expression upon tumour growth in both $CB_2R^{+/+}$ and $CB_2R^{-/-}$ immune cells; however, this decrease was less prominent in CB_2R-lacking cells compared to their CB_2R-expressing counterparts (**Figure R44A**). In T cells specifically, $CB_2R^{-/-}$ cells displayed significantly higher levels of CD69 than $CB_2R^{+/+}$ cells. CD69 expression did not significantly change with tumour progression in CB_2R-positive T cells, while it decreased in their CB_2R-negative counterparts (**Figure R44B**). The overall changes in CD69 expression in total immune cells may be

attributable to changes in its levels in NK and NKT cells. In NK cells, CD69 expression was maximal at T1 and T2 and significantly diminished at T3 in both genotypes. However, this decrease was less pronounced in CB_2R-negative cells, leading to statistically significant differences in large tumours (**Figure R44C**). A very similar pattern was observed in NKT cells, albeit only CB_2R-positive cells showed a significant decrease in CD69 expression at T3, while $CB_2R^{-/-}$ cells maintained stable levels (**Figure R44D**).

These results reinforce the notion that CB_2R may play a significant role in blocking T cell activation during the initial phases of tumour development, while its influence appears to diminish as the disease progresses. By contrast, the immunomodulatory role of CB_2R on NK and NKT cells becomes more evident in advanced tumour stages, where its presence contributes substantially to limiting the activation of these immune cell populations.

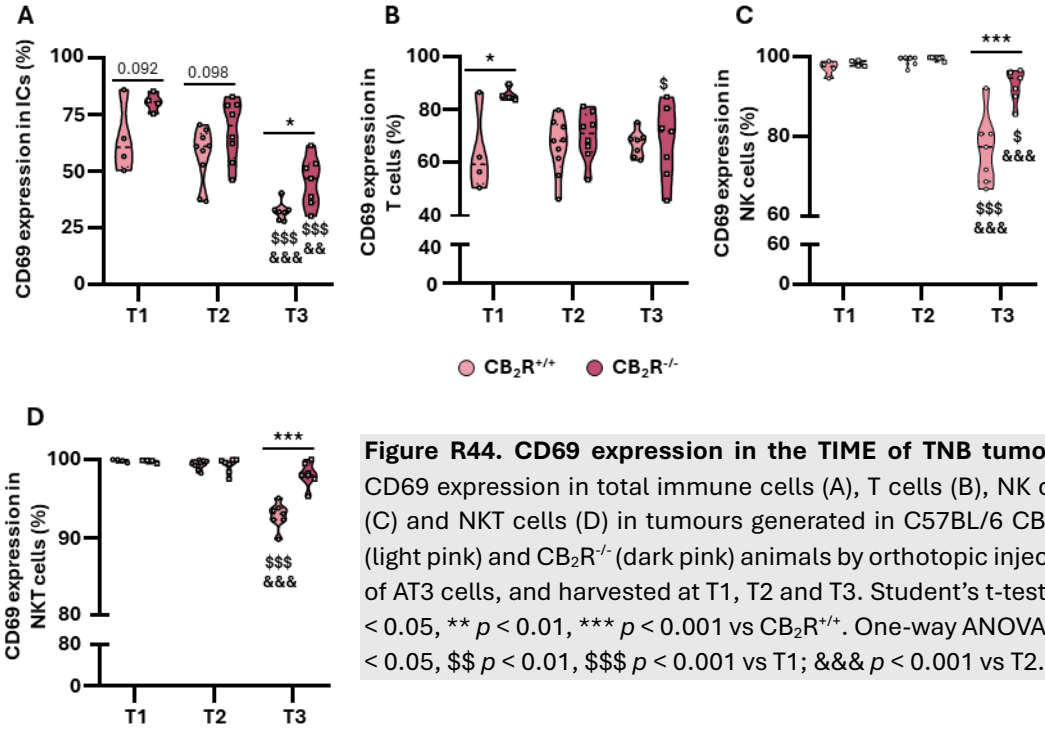

Figure R44. CD69 expression in the TIME of TNB tumours. CD69 expression in total immune cells (A), T cells (B), NK cells (C) and NKT cells (D) in tumours generated in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) animals by orthotopic injection of AT3 cells, and harvested at T1, T2 and T3. Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs $CB_2R^{+/+}$. One-way ANOVA: $ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$ vs T1; &&& $p < 0.001$ vs T2.

The expression of CD107α significantly decreased with tumour progression in both genotypes, although this effect was less pronounced in $CB_2R^{-/-}$ immune cells (**Figure R45A**). In this case, we observed changes in CD107α expression that followed this same pattern (and thereby could account for the observations made in the total immune cell pool) in three immune subpopulations: T, NK and NKT cells. Regarding T cells, CD107α levels where higher in CB_2R-lacking cells at T1 and significantly dropped at T2 and remained relatively stable at T3 both in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ lymphocytes (**Figure R45B**). In NK and NKT cells, CD107a expression was nearly 100 % in small and medium tumours and significantly declined in large tumours for both genotypes. However, this decrease was less pronounced in $CB_2R^{-/-}$ cells, resulting in statistically significant differences in CD107a levels between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ lymphocytes at T3 (**Figures R45C and R45D**).

These results suggest that CB_2R blocks the effector capacity of T cells across all tumour stages, as CB_2R-lacking T cells consistently displayed higher cytotoxic potential than their CB_2R-expressing counterparts. In parallel, the influence of CB_2R signalling on innate

immune cells becomes more prominent in larger tumours, where its expression seems to play a key role in limiting the cytotoxic activity of these cells.

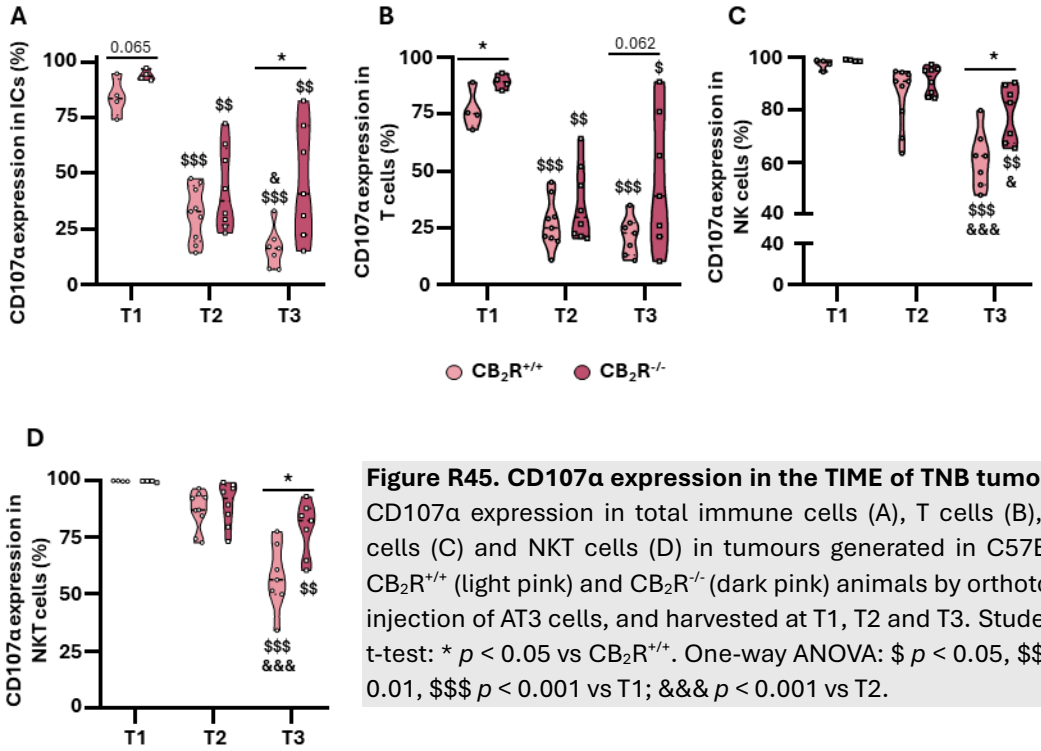

Figure R45. CD107α expression in the TIME of TNB tumours. CD107α expression in total immune cells (A), T cells (B), NK cells (C) and NKT cells (D) in tumours generated in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) animals by orthotopic injection of AT3 cells, and harvested at T1, T2 and T3. Student's t-test: * $p < 0.05$ vs $CB_2R^{+/+}$. One-way ANOVA: \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs T1; &&& $p < 0.001$ vs T2.

Altogether, the results derived from the characterisation of the immune infiltrate and the non-cellular component of the BTME point to a pro-tumoural role of CB_2R contributing to TNBC progression.

ROLE OF TME-CB_2R IN THE GENERATION OF LUNG METASTASES

Distant metastasis remains the leading cause of cancer-related mortality, accounting for approximately 90 % of patient deaths. Specifically in TNBC, only a small percentage of patients with primary disease present distant metastases at diagnosis; however, the majority eventually relapse after treatment, more likely developing distant recurrences (O'Reilly et al., 2021). Since the results described above suggest that CB_2R contributes to TNB tumour progression by promoting the generation of an immunosuppressive TME, we hypothesised that it may also facilitate the establishment of distant metastases. To validate our hypothesis, we employed a lung metastasis model based on the injection of the TNBC AT3 cell line stably expressing firefly luciferase (AT3-luc-mCherry) into the lateral tail vein of 8–10-week-old C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females.

Our results revealed that, at the culling point (six weeks after AT3-luc-mCherry cells injection), 50 % of CB_2R-lacking females were metastasis-free, compared to only 14.3 % in the CB_2R-expressing group (**Figure R46A**). We did not observe significant differences in the time of metastasis onset, although bioluminescence signal was already detected in the lung at week three in 14.3 % of the $CB_2R^{+/+}$ animals, whereas it was first detected at week four in 12.5 % of $CB_2R^{-/-}$ animals. Increasing the sample size may help elucidate if CB_2R-

lacking females indeed develop distant metastasis later than their CB_2R-expressing counterparts. Upon comparing the lungs of animals in which bioluminescence signal was detected during the same week, we found that the number of metastatic lesions was lower in the $CB_2R^{-/-}$ cohort (**Figure R46B**). Although there were no apparent differences in lesion size between the two groups, further experiments will be required to unequivocally determine if CB_2R influences the growth of TNBC cells following their arrival into the lungs.

Together, our findings indicate that a CB_2R-expressing niche facilitates the colonisation of cancer cells and the generation of lung metastases.

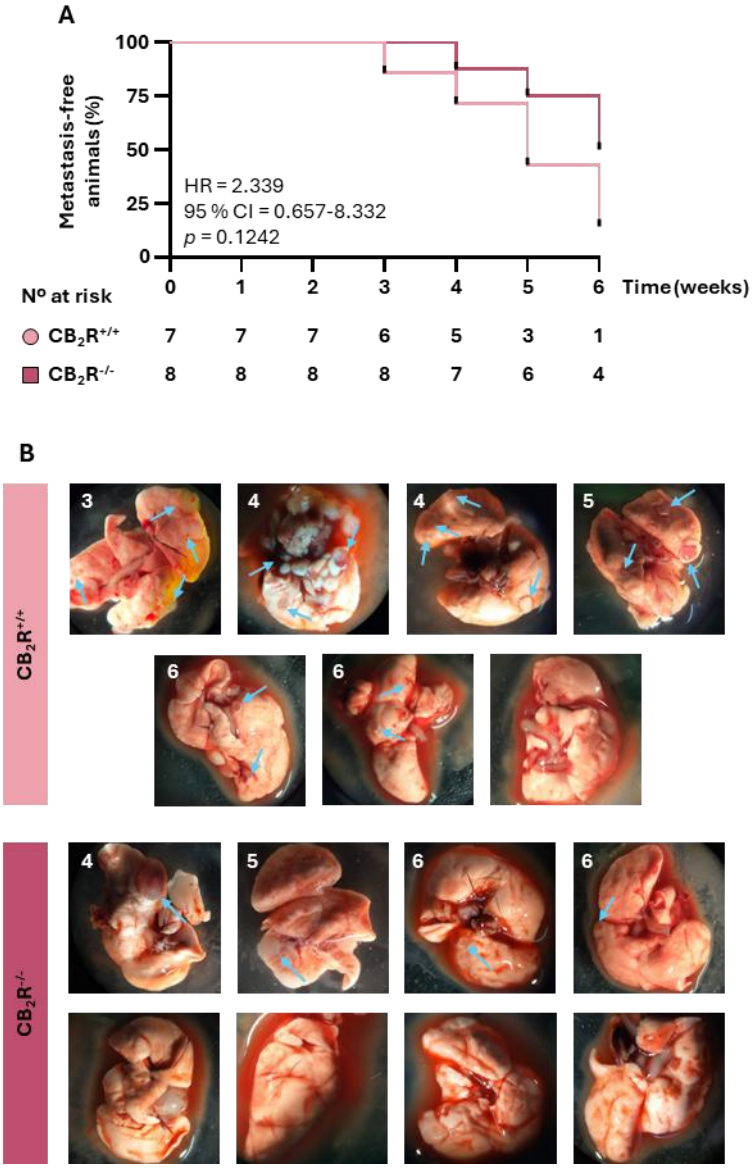

Figure R46. Generation of lung metastasis in an animal model of TNBC. (A) Kaplan-Meier curves showing metastasis onset in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice injected in the lateral vein with the AT3-luc-mCherry cell line. Statistics derived from a Log-rank test. (B) Representative images of lungs from C57BL/6 $CB_2R^{+/+}$ and $CB_2R^{-/-}$ female mice at the culling point, with metastatic lesions pointed by blue arrows. The number displayed on each image corresponds to the week in which metastasis was first detected.

To determine the contribution of CB_2R expressed within the TIME to these observations we evaluated the immune infiltration in the metastatic lesions. Due to technical problems, we could only perform these experiments in 4 of 7 $CB_2R^{+/+}$ animals and 2 of 8 $CB_2R^{-/-}$ animals. Therefore, the small sample size calls for caution when interpreting the results and warrants future experiments to validate them.

We did not observe significant differences in the total pool of infiltrating immune cells between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ animals. Similarly, no changes in the major immune subsets or in T cell subpopulations were found between both genotypes (**Figure R47**). These results suggest that the role of CB_2R in promoting metastasis is likely to be exerted prior to cancer cell colonisation of the secondary organ.

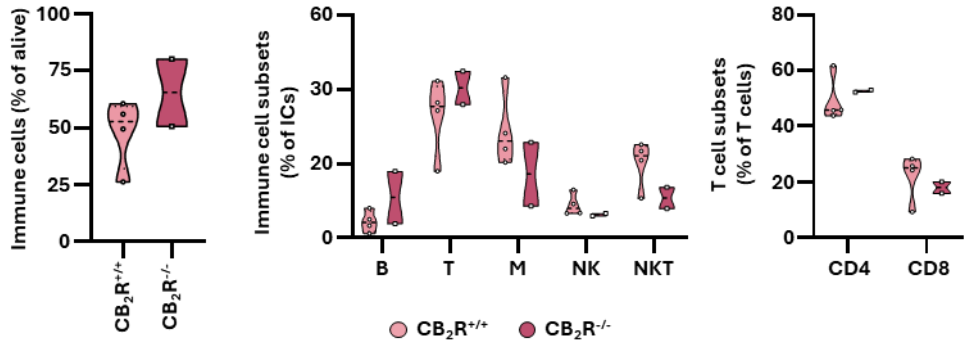

Figure R47. Immune composition of AT3-derived lung metastases. Percentages of total immune cells (left panel), major immune cell subsets (middle panel) and main T cell subpopulations (right panel) infiltrating the metastatic lesions generated in the lungs of C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice by injection in the lateral tail vein of the AT3-luc-mCherry cell line, as assessed by flow cytometry. Student's t-test vs $CB_2R^{+/+}$.

A more detailed phenotypic characterisation of the immune infiltrating cells revealed no significant differences in PD-1 expression, either in the total pool of immune cells or within cytotoxic T cells between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ animals (**Figure R48A**). Similarly, no changes in the levels of PD-L1 were found between genotypes, although there was a trend toward a reduced expression in the absence of CB_2R in the total pool of immune cells. However, this result was not attributable to changes in PD-L1 expression in CD4 T or myeloid cells, as levels were comparable between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ females (**Figure R48B**).

These results indicate that CB_2R may not play a significant role in regulating the PD-1/PD-L1 axis within established metastatic lesions.

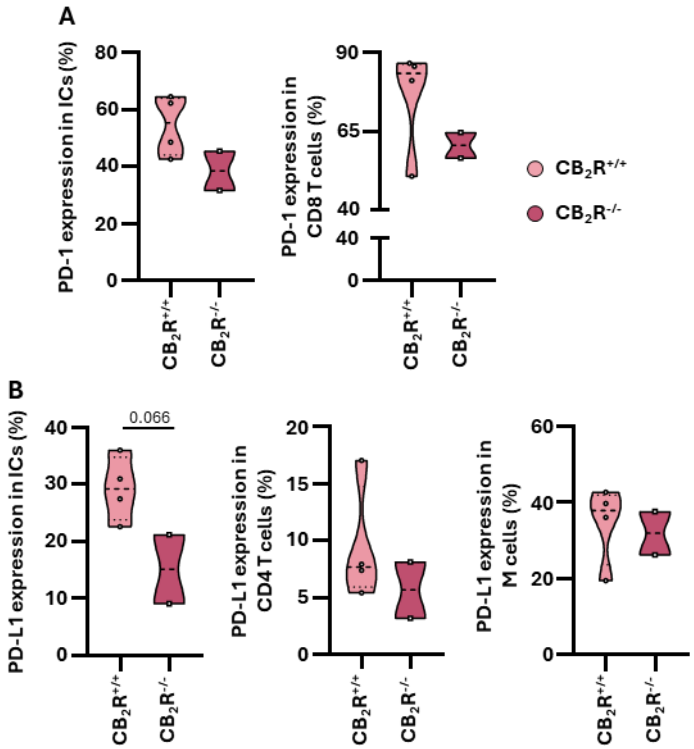

Figure R48. Immune checkpoint expression in immune cells infiltrating lung metastases. (A) Expression of PD-1 in total immune cells (left panel) and cytotoxic T cells (right panel) from lung metastases generated in C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice by injection in the lateral tail vein of the AT3-luc-mCherry cell line, as assessed by flow cytometry. (B) PD-L1 expression in total immune cells (left panel), helper T cells (middle panel) and myeloid cells (right panel) in the same metastatic lesions, as assessed by flow cytometry. Student's t-test vs $CB_2R^{+/+}$.

Regarding the activation status, there was no significant differences in the expression of CD69 in the total infiltrating cells of both genotypes (**Figure R49A**), although there was a significant decrease in the levels of the surface marker on CB_2R-negative T cells compared to their $CB_2R^{+/+}$ counterparts (**Figure R49B**). On the contrary, no changes were detected in NK and NKT cells between both genotypes (**Figures R49C and R49D, respectively**).

These findings suggest that CB_2R does not modulate the activation of innate immune cells in established lung metastases but may facilitate T cell activation at these sites.

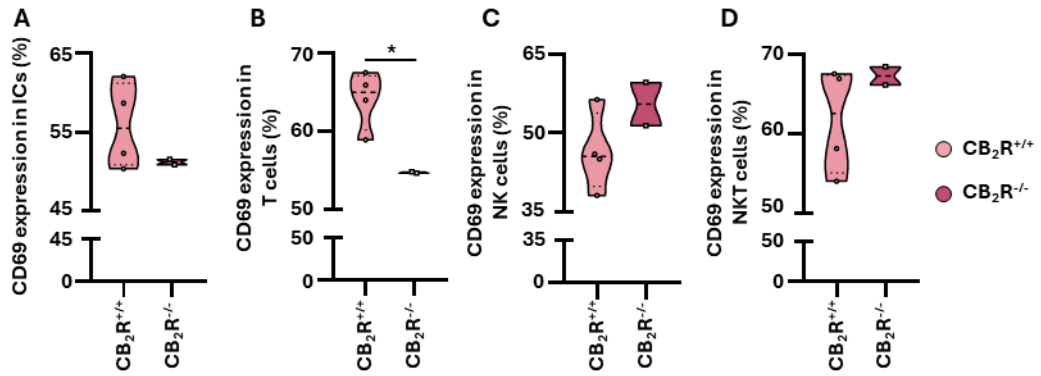

Figure R49. CD69 expression in immune cells infiltrating lung metastases. Expression of the activation marker CD69 in total immune cells (A), T cells (B), NK cells (C) and NKT cells (D) infiltrating the metastatic lesions of C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) females injected in the lateral tail vein with the AT3-luc-mCherry cell line, as assessed by flow cytometry. Student's t-test: * $p < 0.05$ vs $CB_2R^{+/+}$.

Our results also suggest that the cytotoxic capacity of total effector cells was not altered by CB_2R, as the expression of CD107α was similar between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ lesions (**Figure R50A**). Similarly, no differences in the expression of this marker were detectable either in T, NK or NKT cells (**Figure R50B, R50C and R50D,** respectively).

These observations imply that CB_2R may not significantly influence the effector activity of NK, NKT or T cells within established metastatic lesions.

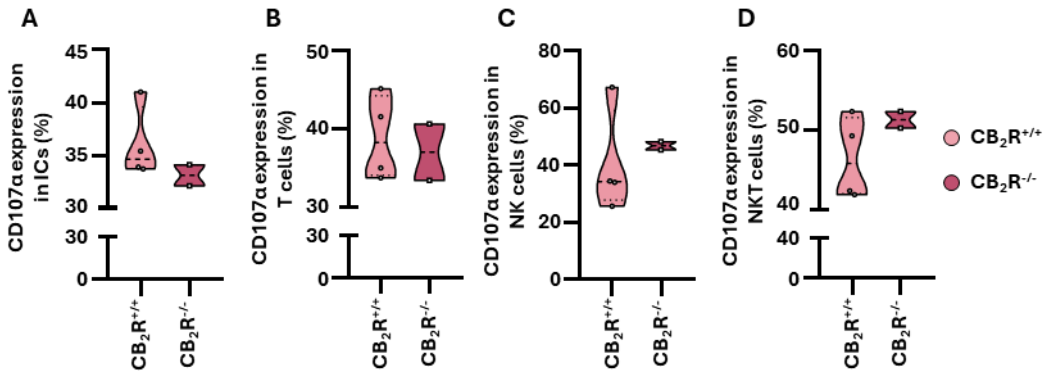

Figure R50. CD107α expression in immune cells infiltrating lung metastases. Expression of the activation marker in total immune cells (A), T cells (B), NK cells (C) and NKT cells (D) infiltrating the metastatic lesions of C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) females injected in the lateral tail vein with the AT3-luc-mCherry cell line, as assessed by flow cytometry. Student's t-test vs $CB_2R^{+/+}$.

Collectively, these results indicate that CB_2R expressed by some unidentified cells (other than cancer cells) plays a pro-metastatic role by promoting tumour cell colonisation of the lung. In addition, our results suggest that, once the lung metastasis lesions have been established, the role of CB_2R in the main immune cell populations may be irrelevant. Therefore, we hypothesise that the impact of CB_2R on metastatic progression is more likely to occur prior to tumour cell colonisation of distant organs. This could be mediated by the

generation of a more favourable pre-metastatic niche, by impairing immune surveillance at extra-pulmonary sites, or by the contribution of other non-immune cell types to the enhanced migratory capacity of tumour cells.

AIM 3. ANALYSIS OF THE SIMULTANEOUS TARGETING OF CB_2R IN IMMUNE AND CANCER CELLS

The results presented in the previous sections of this thesis indicate that CB_2R is expressed within the BTIME and that its expression plays a pro-tumoural and pro-metastatic role, particularly in TNBC. This BC subtype is considered the most aggressive and is associated with the poorest prognosis. However, the introduction into clinical practice of therapies aimed to reactivating the patient's immune response against the tumours (i.e., immunotherapies) has led to improved outcomes. Unfortunately, the proportion of TNBC patients that respond to this therapeutic approach is still very limited, which warrants a deeper knowledge on the role of the BTIME to cancer progression. Although CB_2R activation by cannabinoids has demonstrated robust antitumour responses in preclinical models of BC (Blasco-Benito et al., 2018; Caffarel et al., 2010), it has also been shown to produce immunosuppressive effects (Lombard et al., 2007; Massi et al., 2000; M. Yuan et al., 2002), which may be a reason to contraindicate their use in cancer patients—particularly those receiving immunotherapy.

In this context, the third objective of this thesis was to investigate the balance between the antitumour and immunosuppressive effects of CB_2R activation in models of BC where tumour cells and immune cells coexist, thereby advancing our understanding of the complex interplay between cannabinoid signalling and immune regulation in BC.

ASSESSMENT OF THE IMPACT OF CB_2R ACTIVATION ON IMMUNE CELL FUNCTIONS

In a perfectly coordinated process, naive T cells continuously recirculate through the body, entering secondary lymphoid organs, such as the tumour-draining lymph nodes, where they encounter APCs displaying tumour-associated antigens. Upon its recognition in the presence of co-stimulatory signals, T cells differentiate into effector cells and migrate to the tumour, where they recognise and eliminate cancer cells (CD8 T cells) or orchestrate immune responses (CD4 T cells).

The results obtained in the previous chapters of this thesis showed that CB_2R is expressed by different immune cell populations in the spleen and in the BTME, which led us to hypothesise that cannabinoids may be able to modulate their function at key stages of tumour surveillance: before, during and after recognition of the cognate antigen presented by an APC, as well as during interaction with tumour cells. To investigate the consequences of CB_2R activation in these scenarios, we employed different treatment models (each outlined in the corresponding section) and tested two different cannabinoid compounds: HU-308 (a CB_2R selective agonist), and THC (a mixed CB_1R/CB_2R agonist).

To choose the most appropriate cannabinoid concentrations for our studies, we first challenged cancer cells with increasing concentrations of the two selected compounds. As previously reported by our group for other TNBC cell lines (Blasco-Benito et al., 2018), both

HU-308 and THC decreased AT3 cell viability in a concentration-dependent manner, with HU-308 exhibiting greater potency than THC (IC_{50} 10.87 µM and 23.25 µM, respectively) (**Figure R51**).

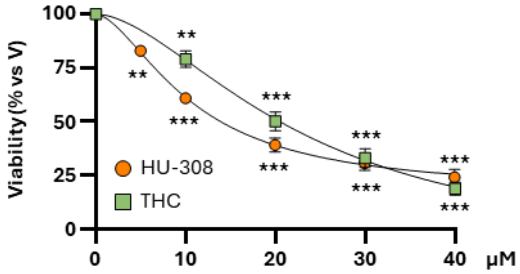

Figure R51. Effect of cannabinoids on BC cell viability. Viability of AT3 BC cells, as assessed by crystal violet assay, after a three-day treatment with increasing concentrations of HU-308 or THC. One-way ANOVA: ** $p < 0.01$; *** $p < 0.001$ vs vehicle-treated AT3 cells.

Based on these results, we selected two concentrations of each cannabinoid to assess the consequences of CB_2R targeting in immune cells: one below and one above the IC_{50} (5 and 15 µM for HU-308 and 10 and 30 µM for THC, respectively).

EFFECT OF CANNABINOIDS ON IMMUNE CELLS

EFFECT OF CANNABINOIDS ON IMMUNE CELLS BEFORE ACTIVATION

Immunosurveillance is a continuous process in which naive T cells continuously recirculate throughout the body in search of their cognate antigen. During this journey, these cells may encounter cannabinoids—whether through medicinal or recreational use—which could potentially alter their responsiveness to critical stimuli required for their proliferation and differentiation. To investigate the impact of cannabinoids on the activation capacity of naive immune cells (i.e., prior to the initiation of T cell activation programs), we treated freshly isolated splenocytes obtained from our C57BL/6 and CB_2R^{eGFP} animal models with cannabinoids for 24 h, and then challenged these cells with and stimulus for a further 24 h, after which we performed flow cytometry analysis (**Figure R52**).

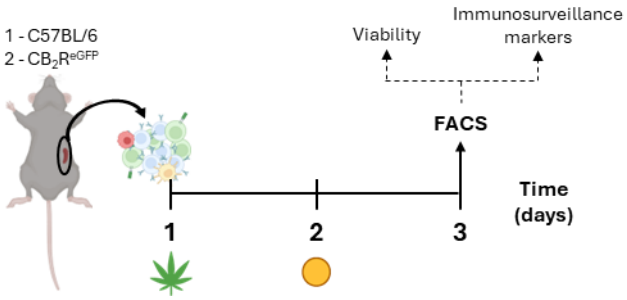

Figure R52. Experimental set up. Schematic representation of the strategy used to assess the effect of CB_2R targeting before immune cell activation. Splenocytes were treated with cannabinoids (marijuana leaf icon) for 24 h and subsequently activated with ConA (yellow sphere icon) for 24 h prior to flow cytometry (FACS) analysis.

Effects on splenocytes isolated from the C57BL/6 model

Preliminary results (n = 1) in the C57BL/6 model showed that both HU-308 and THC decreased the viability of CB_2R-expressing immune cells in a concentration-dependent manner. Unexpectedly, a virtually identical response was observed in cells lacking CB_2R (**Figure R53**), pointing to a CB_2R-independent mechanism of action.

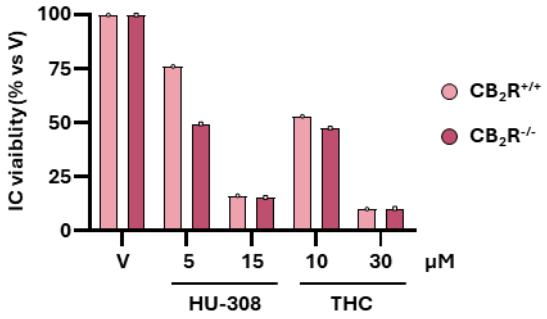

Figure R53. Effect of cannabinoids on the viability of splenocytes obtained from the C57BL/6 model. Viability, as assessed by flow cytometry, of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and cultured for 24 h in the presence of cannabinoids and activated for further 24 h with ConA. Viability was relativised to that of the vehicle-treated (V) group, that was set as 100.

Cannabinoids also increased the levels of the early activation marker CD69 in both total immune cells and T cells. Consistent with the viability assays, no differences were detected between cells expressing or lacking CB_2R (**Figure R54**), again pointing to a CB_2R-independent mechanism. Although this experiment was performed just once and should therefore be interpreted with caution, the suggest that cannabinoids promote signalling pathways leading to CD69 expression.

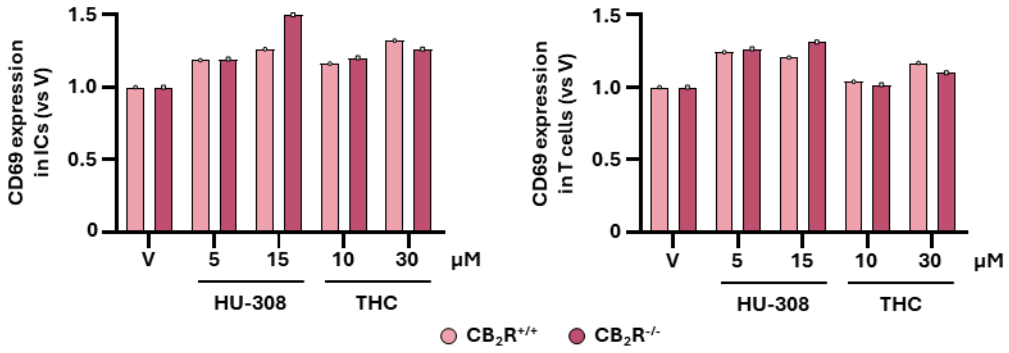

Figure R54. Effect of cannabinoids on the T cell activation marker CD69. Expression of CD69, as assessed by flow cytometry, in total immune cells (left panel) and T cells (right panel) isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and cultured for 24 h in the presence of cannabinoids and activated for further 24 h with ConA. CD69 expression was relativised to that of the vehicle-treated (V) group, that was set as 1.

Taken together, these results suggest that cannabinoids decrease immune cell viability and alter early signalling events implicated in immune cell activation independently of CB_2R activation, at least in this experimental context.

Effects on splenocytes isolated from the CB_2R^{eGFP} model

We performed similar experiments in splenocytes obtained from the CB_2R^{eGFP} model, in which cells expressing and lacking CB_2R coexist within the same sample. This system enables discrimination of these two populations based on GFP expression, allowing a direct assessment of their specific contribution to the overall effect.

We first confirmed that immune cell viability decreased in a concentration-dependent manner following cannabinoid treatment (**Figure R55A**), and that this effect was attributable to a reduction in the viability of both GFP^+ (CB_2R^+) and GFP^- (CB_2R^-) cells (**Figure R55B**), pointing to a CB_2R-independent mechanism as in the C57BL/6 model.

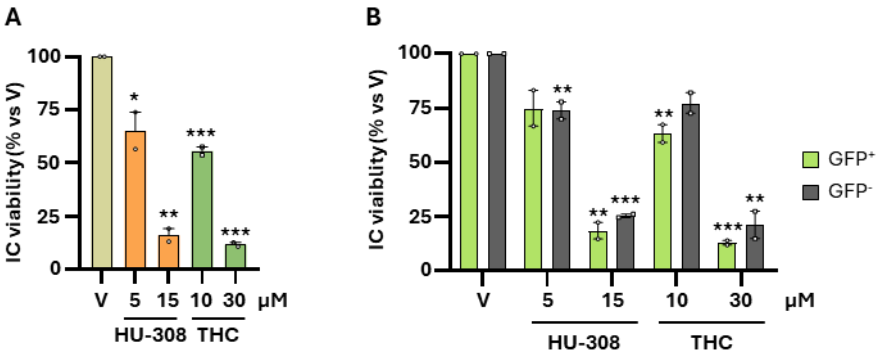

Figure R55. Effect of cannabinoids on the viability of splenocytes obtained from the CB_2R^{eGFP} model. Overall immune cell viability (A) or stratified according to GFP (CB_2R) expression (B) of splenocytes isolated from CB_2R^{eGFP} female mice and cultured for 24 h in the presence of cannabinoids and activated for further 24 h with ConA. Viability, assessed by flow cytometry, was relativised to that of the vehicle-treated (V) group, that was set as 100. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

We then analysed the expression of other activation marker, the immune checkpoint PD-1. In this context, both compounds showed opposite actions: while none of them showed any effect at the lower concentration, the selective CB_2R agonist upregulated, whereas the CB_1R/CB_2R mixed agonist downregulated PD-1 in total immune cells and T cells (**Figures R56A and R56C,** respectively). Strikingly, these effects were CB_2R independent as they were produced both in cells expressing (GFP^+) and lacking (GFP^-) this receptor (**Figures R56B and R56D**).

Collectively, these findings suggest that cannabinoids decrease immune cell viability and impair their subsequent ability to respond to an activating stimulus in a CB_2R-independent mechanism.

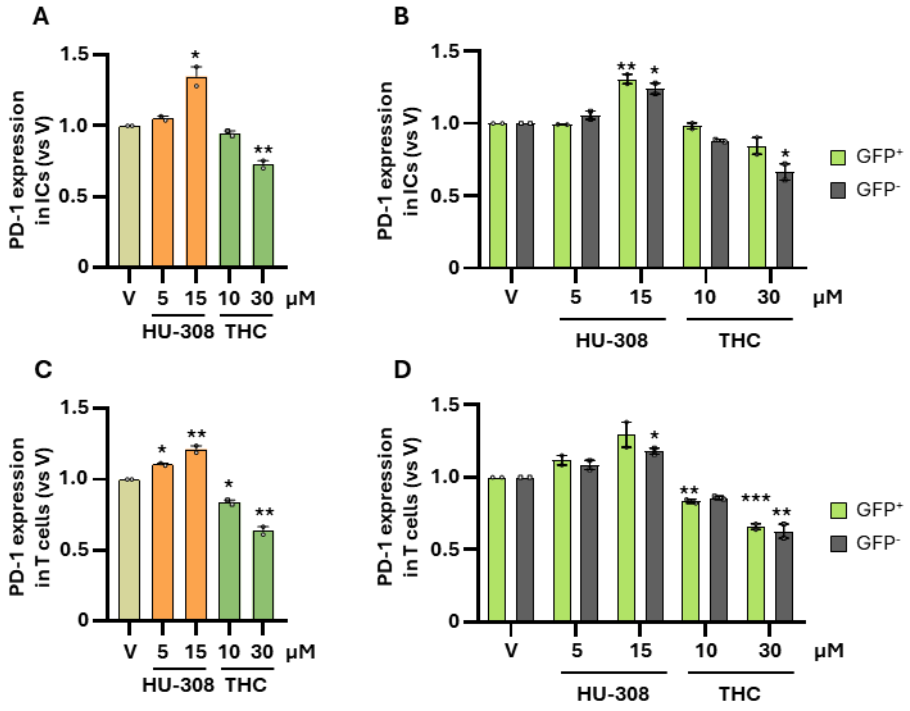

Figure R56. Effect of cannabinoids on the activation marker PD-1. Overall PD-1 expression (A and C) or stratified according to GFP (CB_2R) expression (B and D) in total immune cells (A-B) or T cells (C-D) isolated from CB_2R^{eGFP} female mice and cultured for 24 h in the presence of cannabinoids and activated for further 24 h with ConA. PD-1 expression, assessed by flow cytometry, was relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

EFFECT OF CANNABINOIDS ON IMMUNE CELLS DURING ACTIVATION

During antigen recognition in the tumour-draining lymph node, T cells undergo activation and clonal expansion. Exposure to cannabinoids—whether through medical prescription or recreational use—at this critical stage of tumour immunosurveillance may interfere with the signalling pathways required for optimal T cell activation, potentially dampening the immune response. To evaluate the consequences of the pharmacological targeting of CB_2R in immune cells undergoing activation, we concomitantly treated splenocytes from our C57BL/6 and CB_2R^{eGFP} animal models with cannabinoids and ConA for the indicated time. Subsequently, we performed different experimental procedures as indicated in **Figure R57**.

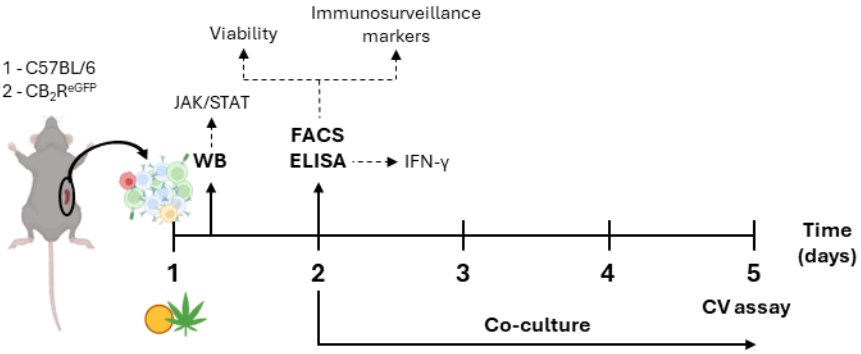

Figure R57. Experimental set up. Schematic representation of the strategy used to assess the effect of CB_2R targeting during immune cell activation. Splenocytes were concomitantly treated with ConA (yellow sphere icon) and cannabinoids (marijuana leaf icon) for 6 h for Western blot (WB) analysis or for 24 h for flow cytometry (FACS), ELISA and co-culture assays. BC cell viability assessed by crystal violet (CV) assay was used as a readout for the killing capacity of immune cells.

Effects on splenocytes isolated from the C57BL/6 model

Flow cytometry analysis revealed a significant, concentration-dependent decrease in splenocyte viability in response to both cannabinoids. To our surprise, but in line with the results obtained in the previous model, no differences between the two genotypes were observed, indicating that the effect was not mediated by selective activation of CB_2R (**Figure R58**).

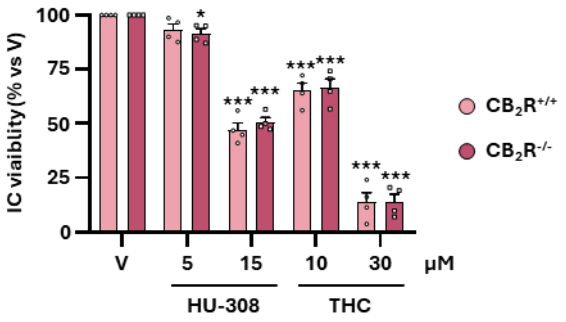

Figure R58. Effect of cannabinoids on the viability of splenocytes obtained from the C57BL/6 model. Viability, as assessed by flow cytometry, of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and cultured for 24 h in the presence of ConA and cannabinoids. Viability was relativised to that of the vehicle-treated (V) group, that was set as 100. One-way ANOVA: * $p < 0.05$, *** $p < 0.001$ vs vehicle-treated cells.

In contrast to what was observed in the previous experimental model, none of the concentrations tested affected the expression of the activation marker CD69, either in the total population or specifically in T cells (**Figure R59A**).

Conversely, PD-1 levels were modulated by cannabinoids in a biphasic mode. At the lowest concentrations tested, they did not modify PD-1 expression in CB_2R-expressing cells, but they produced a significant increase in PD-1 expression in the total pool of CB_2R-lacking immune cells. This effect was not due to increased expression in T cells as the levels of this marker remained unchanged in this cell population. At the highest concentrations, both cannabinoids significantly reduced PD-1 surface expression in total immune cells and T cells, which may indicate a decreased activation of the cells, in both genotypes, once again pointing to a CB_2R-independent effect (**Figure R59B**).

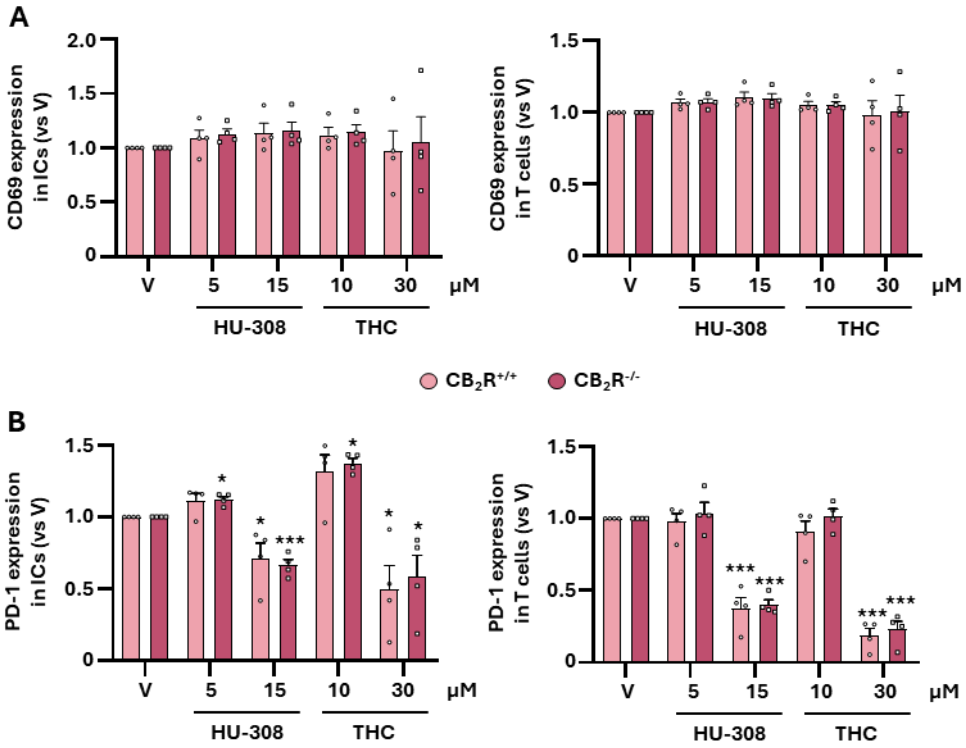

Figure R59. Effect of cannabinoids on the activation markers CD69 and PD-1. Expression of CD69 (A) and PD-1 (B), as assessed by flow cytometry, in total immune cells (left panels) and T cells (right panels) isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and cultured for 24 h in the presence of ConA and cannabinoids. CD69 and PD-1 expression were relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, *** $p < 0.001$ vs vehicle-treated cells.

The analysis of the degranulation marker CD107α on the surface of immune cells did not support the notion that cannabinoids inactivate them (**Figure R60**). Thus, CD107α levels did not change in total immune cells and CB_2R-expressing T cells following cannabinoid treatment. However, a significant increase was observed in total immune cells lacking CB_2R. This effect was not reproduced when specifically analysing T cells, in which CD107α expression remained unchanged, pointing to other immune cell types as the responsible for the observed changes in CD107α levels in the total population. In any case, CD107α levels were minimal compared to those observed in immune cells from the BTIME, suggesting that the detected differences may have limited biological relevance. A complementary analysis of the cytolytic granule composition could help clarify this issue.

Overall, our results indicate that cannabinoid exposure during immune cell activation reduces viability, while their impact on functional activity remains unclear due to conflicting data results. Importantly, in this experimental model, the effects of cannabinoids appear to be independent of CB_2R activation.

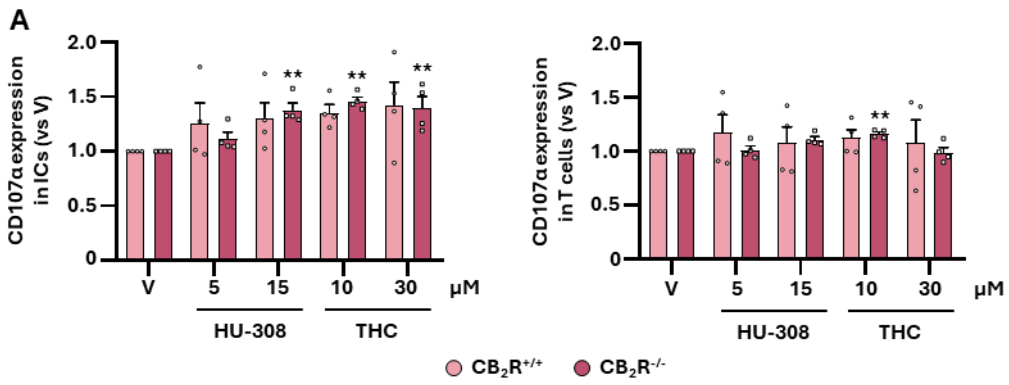

Figure R60. Effect of cannabinoids on the cytotoxic marker CD107α. Expression of CD107α, as assessed by flow cytometry, in total immune cells (left panel) and T cells (right panel) isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and cultured for 24 h in the presence of ConA and cannabinoids. CD107α expression was relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: ** $p < 0.01$ vs vehicle-treated cells.

Next, we investigated whether cannabinoids modulate the production of soluble factor by immune cells that could influence tumour cells. We focused on IFN-γ, a cytokine with well-established antitumour activity.

ELISA analysis of supernatants from $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes concomitantly treated with cannabinoids and ConA revealed a concentration-dependent reduction in IFN-γ production, that was not mediated by CB_2R activation as it occurred in both genotypes (**Figure R61**). To rule out the possibility that the reduced levels of IFN-γ resulted from a decreased immune cell viability (and therefore a reduction in the number of producer cells), IFN-γ release was normalised to cell viability, as assessed by flow cytometry (**Figure R59**), which confirms that the observed effect was due to an impaired cytokine production by viable cells. Therefore, cannabinoids not only reduced the percentage of IFN-γ-producing cells but also impaired the effector capacity of the remaining viable cells regardless of CB_2R status.

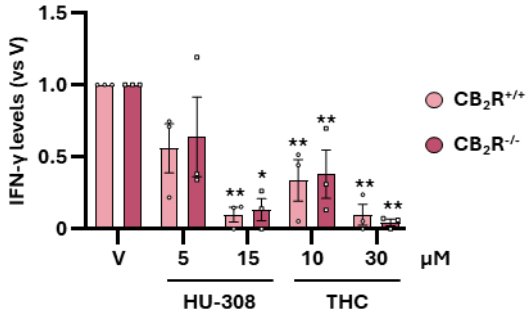

Figure R61. Relative IFN-γ production by splenocytes obtained from the C57BL/6 model. IFN-γ release was assessed by ELISA in the supernatant of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) mice and cultured for 24 h in the presence of ConA and cannabinoids. Values were first relativised to cell viability, as determined by flow cytometry, and then to the production observed in the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ vs vehicle-treated cells.

We then assessed the status of the JAK/STAT cascade, which is crucial for effector T cell functions and has been shown to be regulated by CB_2R in T cells when cannabinoids are added before activation (X. Xiong et al., 2022). By Western blot (**Figure R62**), we first confirmed that treatment with ConA significantly increased the phosphorylation of STAT1 in

both $CB_2R^{+/+}$ and $CB_2R^{-/-}$ immune cells ($p < 0.01$ vs basal). Interestingly, ConA also significantly enhanced the total levels of STAT1 in CB_2R-lacking cells ($p < 0.05$ vs basal), with no such effect observed in CB_2R-expressing cells. In contrast, neither STAT3 total levels nor its phosphorylation were altered by ConA treatment in either genotype. Surprisingly, PTP1B and TC-PTP phosphatases were not upregulated in response to the activating stimulus; moreover, TC-PTP was reduced in CB_2R-deficient cells ($p = 0.0763$ vs basal), potentially indicating a greater activation capacity in these cells.

Regarding cannabinoid treatment of non-activated immune cells, both HU-308 and THC significantly decreased the phosphorylation of STAT1 and STAT3 in a CB_2R-independent fashion ($p < 0.01$ vs basal), with no differences between genotypes. No changes were detected in the total expression of STAT1 or STAT3 in CB_2R-expressing cells. Notably, treatment with HU-308 of CB_2R-lacking immune cells resulted in a trend towards increased expression of STAT1 ($p = 0.0849$ vs basal), and a significant upregulation of STAT3 ($p < 0.05$ vs basal). Regarding the negative regulators, THC significantly decreased PTP1B levels in CB_2R-expressing immune cells ($p < 0.05$ vs basal), while HU-308 significantly decreased TC-PTP expression in $CB_2R^{-/-}$ immune cells ($p < 0.05$ vs basal), without affecting PTP1B.

In stimulated immune cells, both HU-308 and THC significantly reduced ConA-induced STAT1 phosphorylation in CB_2R-expressing and CB_2R-lacking splenocytes ($p < 0.05$ vs ConA-activated). A similar effect was observed for STAT3: although ConA stimulation alone did not affect STAT3 phosphorylation, cannabinoid treatment further decreased it ($p = 0.0568$ for $CB_2R^{+/+}$ and $p < 0.05$ for $CB_2R^{-/-}$ cells vs ConA-activated). While THC did not affect the total STAT protein levels, HU-308 increased total STAT1 levels in $CB_2R^{+/+}$ immune cells ($p < 0.05$ vs ConA-activated) and total STAT3 levels in $CB_2R^{-/-}$ immune cells ($p = 0.0971$ vs ConA-activated). Neither HU-308 nor THC significantly modified PTP1B or TC-PTP levels in either genotype.

Although these results are complex, we speculate that cannabinoids, via a CB_2R-independent mechanism, suppress basal JAK/STAT signalling and impair its activation in response to stimulation. Further experiments will be required to understand the physiological significance and the underlying mechanism responsible for this effect.

Together, our findings indicate that cannabinoids, through a CB_2R-independent mechanisms, exert immunosuppressive-like effects on immune cells during activation—manifested as reduced cell viability, signs of diminished activation, impaired IFN-γ production, and blockade of the JAK/STAT signalling pathway in response to stimulation.

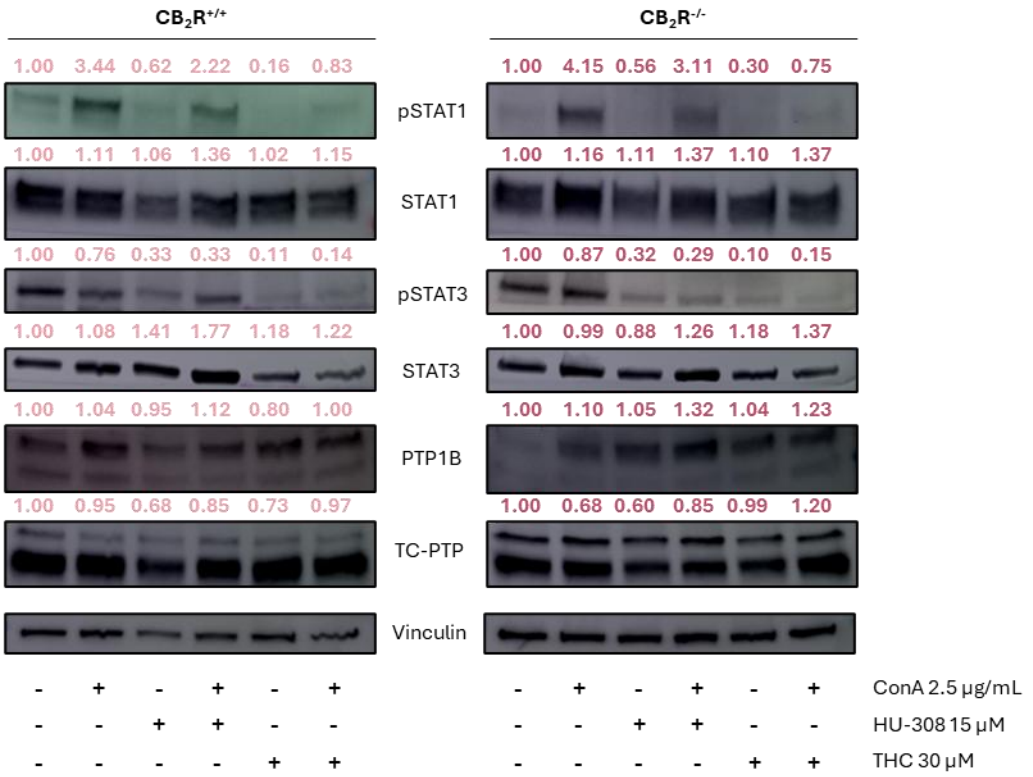

Figure R62. Analysis of the JAK/STAT cascade in splenocytes obtained from the C57BL/6 model. Representative Western blots of the indicated JAK/STAT signalling pathway elements and the negative regulator phosphatases in cultures of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice, treated for 6 h in the presence of the indicated compounds. Densitometric values were first normalised to Vinculin or total protein and then to the vehicle-treated group, that was set as 1. The mean value of 2-4 independent experiments is shown above each membrane slice. Student's t-test vs vehicle-treated or ConA-activated.

Finally, we sought to determine whether the effect of cannabinoids on immune cells translated into an altered cytotoxic capacity against cancer cells. To this aim, we performed cell viability assays in co-cultures of splenocytes and AT3 tumour cells. In the C57BL/6 model, $CB_2R^{+/+}$ splenocytes showed a concentration-dependent reduction in the killing capacity in response to both cannabinoids, whereas in $CB_2R^{-/-}$ splenocytes this was only observed with THC (**Figure R63A**).

To determine whether these results were attributable to the action of cannabinoids on T cells specifically, we repeated the same experiments using cells isolated from CB_2R^{eGFP} and CD4-$CB_2R^{-/-}$ female mice. Consistent with previous results, both cannabinoids reduced the cytotoxic activity of CB_2R-expressing immune cells, whereas only THC impaired the killing capacity of CB_2R-lacking T cells (**Figure R63B**).

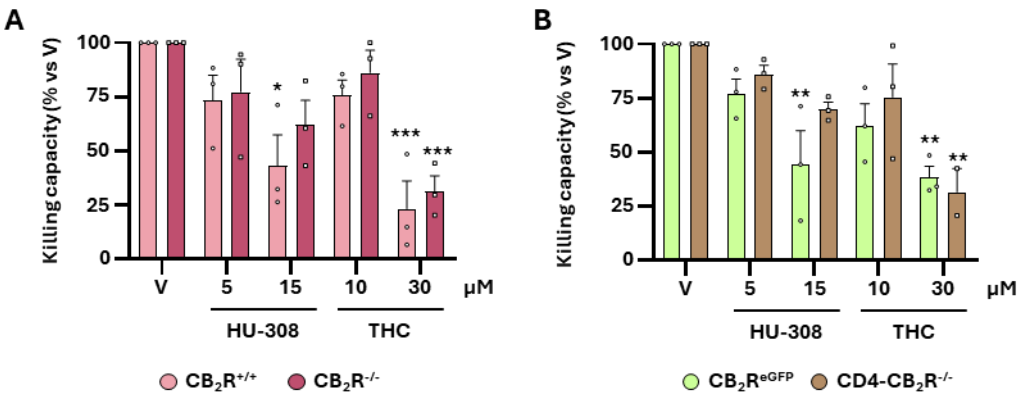

Figure R63. Analysis of the killing capacity of splenocytes obtained from the C57BL/6 and CB_2R^{eGFP} models. Killing capacity of (A) C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) or (B) CB_2R^{eGFP} (green) and CD4-$CB_2R^{-/-}$ (brown) splenocytes cultured for 24 h in the presence of ConA and cannabinoids, as assessed in co-cultures of these cells with AT3 cancer cells (ratio E:T 50:1) and determined as 100 minus the viability (%) of AT3 cells. These values were then relativised to that of the vehicle-treated (V) group, that was set as 100. Two-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

Taken together, these results indicate that cannabinoids reduce the cytotoxic capacity of multiple immune cells—not only T cells—through a CB_2R-independent mechanism.

Effects on splenocytes isolated from the CB_2R^{eGFP} model

To further study the impact of cannabinoids treatment during activation, we performed flow cytometry experiments using splenocytes isolated from C57BL/6 CB_2R^{eGFP} females.

Again, cannabinoids elicited a significant, concentration-dependent reduction in immune cell viability (**Figure R64A**), which was attributed to decreased viability of both GFP^+ (CB_2R^+) and GFP^- (CB_2R^-) populations (**Figure R64B**).

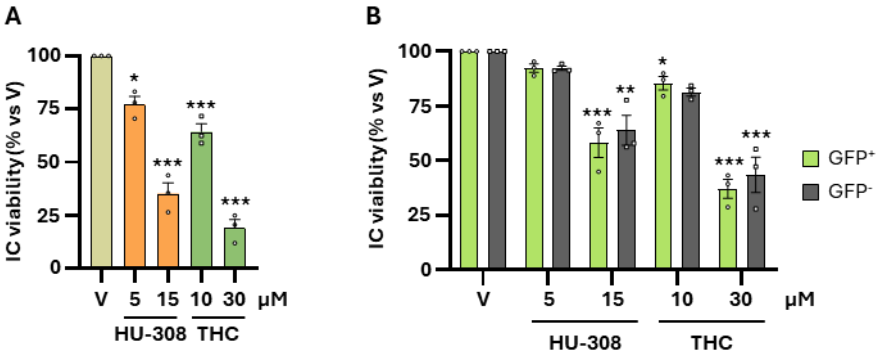

Figure R64. Effect of cannabinoids on the viability of splenocytes obtained from the CB_2R^{eGFP} model. Overall immune cell viability (A) or stratified according to GFP (CB_2R) expression (B) of splenocytes isolated from CB_2R^{eGFP} female mice and cultured for 24 h in the presence of ConA and cannabinoids. Viability, assessed by flow cytometry, was relativised to that of the vehicle-treated (V) group, that was set as 100. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

Flow cytometry analyses showed that PD-1 expression was not modified by cannabinoids in the total pool of immune cells (**Figure R65A**), whereas it was downregulated in T cells (**Figure R65C**), suggesting that the expression of the immune checkpoint might be increased in other immune subsets. When stratifying by CB_2R expression, we found that—as in the C57BL/6 model—the dynamic changes induced by cannabinoids differed depending on CB_2R expression: in the total immune cell pool, PD-1 levels remained unchanged in GFP^+ (CB_2R^+) cells, whereas GFP^- (CB_2R^-) cells displayed reduced PD-1 expression (**Figure R65B**). In the specific case of T cells, both cannabinoids decreased PD-1 expression regardless of CB_2R status (**Figure R65D**).

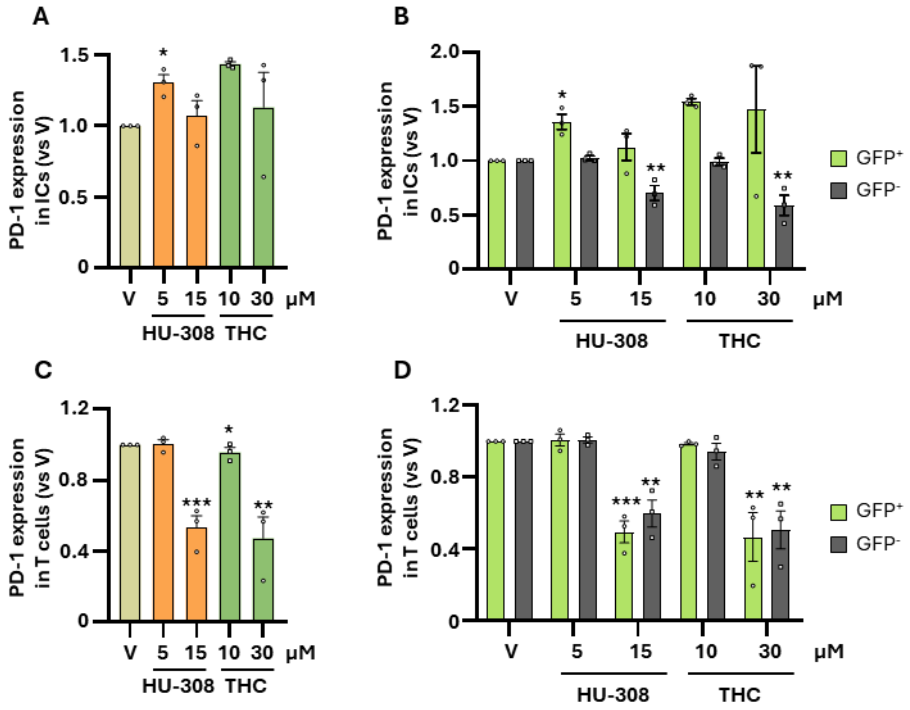

Figure R65. Effect of cannabinoids on the activation marker PD-1. Overall PD-1 expression (A and C) or stratified according to GFP (CB_2R) expression (B and D) in total immune cells (A-B) or T cells (C-D) isolated from CB_2R^{eGFP} female mice and cultured for 24 h in the presence of ConA and cannabinoids. PD-1 expression, assessed by flow cytometry, was relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

These results suggest that cannabinoids decrease immune cell viability and impair their activation through a CB_2R-independent mechanism, which is in line with the observations made in the C57BL/6 model.

EFFECT OF CANNABINOIDS ON IMMUNE CELLS AFTER ACTIVATION

Following recognition of their cognate antigen in the presence of costimulatory signals, T cells differentiate into effector cells that orchestrate adaptative immune responses. Exposure to cannabinoids at this stage—whether through medical or recreational use—

could potentially modulate the signalling pathways and functional outcomes of T cell activation, therefore influencing the efficacy of immunosurveillance mechanisms. To evaluate the impact of CB_2R activation on ongoing immune responses, freshly isolated splenocytes from C57BL/6 and CB_2R^{eGFP} animal models were first activated with ConA for 24 h and then treated with cannabinoids for the indicated time. Subsequently, we performed different experimental procedures as indicated in **Figure R66.**

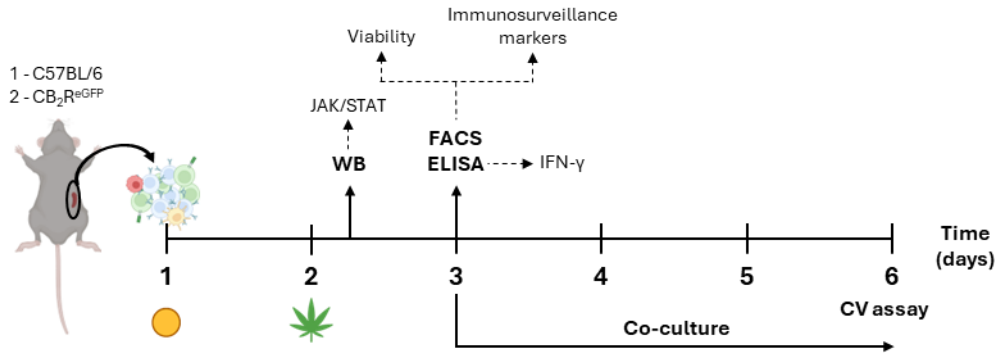

Figure R66. Experimental set up. Schematic representation of the strategy used to assess the effect of cannabinoid treatment administered after immune cell activation. Isolated splenocytes were activated for 24 h with ConA (yellow sphere icon) and subsequently treated with cannabinoids (marijuana leaf icon) for 6 h for Western blot (WB) analyses or for 24 h for flow cytometry (FACS), ELISA and co-culture assays. BC cell viability assessed by crystal violet (CV) assay was used as a readout for the killing capacity of immune cells.

Effects on splenocytes isolated from the C57BL/6 model

Flow cytometry analysis of immune cell viability showed a concentration-dependent reduction in response to HU-308 and THC. This effect was virtually identical in CB_2R-expressing and CB_2R-lacking cells (**Figure R67**), again pointing to a CB_2R-independent mechanism.

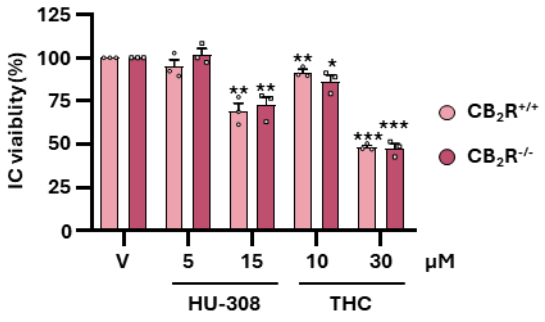

Figure R67. Effect of cannabinoids on the viability of splenocytes obtained from the C57BL/6 model. Viability, as assessed by flow cytometry, of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and activated for 24 h with ConA followed by culture in the presence of cannabinoids for another 24 h. Viability was relativised to that of the vehicle-treated (V) group, that was set as 100. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

In contrast to previous results during activation, PD-1 expression largely remained unchanged following cannabinoid treatment. The only exception was a slight increase in the total immune population in response to THC, which was CB_2R-independent as it occurred in both genotypes, and was not attributable to T cells, since PD-1 expression in this subset

was not increased; instead, T cells showed a slight reduction in PD-1 levels (**Figure R68**). These results suggest that cannabinoids do not reverse the activated phenotype of T cells once it has been established.

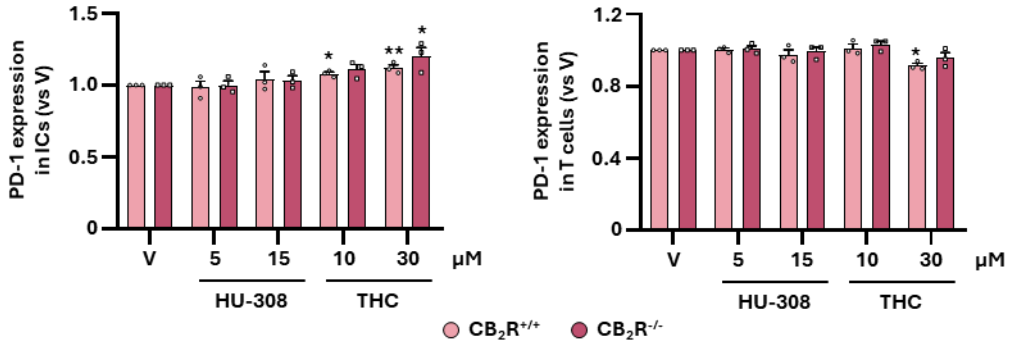

Figure R68. Effect of cannabinoids on the activation marker PD-1. Expression of PD-1, as assessed by flow cytometry, in total immune cells (left panels) and T cells (right panels) isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice and activated for 24 h with ConA followed by culture in the presence of cannabinoids for another 24 h. PD-1 expression was relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$ vs vehicle-treated cells.

We then evaluated IFN-γ production in the supernatant of cannabinoid-treated splenocytes by ELISA. Again, cytokine production was relativised to cell viability (**Figure R67**) to determine whether the observed effect was due to reduce number of producer cells or to affected effector capacity. We did not observe differences in IFN-γ release with either compound at any concentration tested (**Figure R69**). These results indicate that, although the proportion of cytokine-producing cells was reduced, the effector capacity of the remaining viable immune cells was preserved.

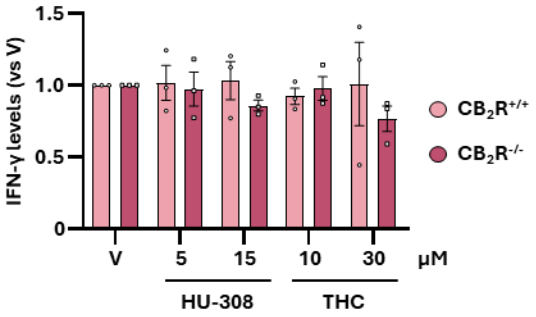

Figure R69. Relative IFN-γ production by splenocytes obtained from the C57BL/6 model. IFN-γ release was assessed by ELISA in the supernatant of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) mice activated for 24 h with ConA followed by culture in the presence of cannabinoids for another 24 h. Values were first relativised to cell viability as determined by flow cytometry, and then to the production observed in the vehicle-treated group, that was set as 1. One-way ANOVA vs vehicle-treated cells.

We next examined the JAK/STAT signalling pathway by Western blot analysis of key components (**Figure R70**). ConA stimulation increased total STAT1 levels and its phosphorylation ($p < 0.05$ and $p = 0.0561$ vs basal, respectively) in $CB_2R^{+/+}$ immune cells. Similar effects were observed for $CB_2R^{-/-}$ immune cells: both STAT1 expression and phosphorylation were significantly increased in ConA-treated cells ($p < 0.01$ vs basal). Conversely, STAT3 levels remained largely unchanged upon stimulation, albeit a trend

toward increased expression was noted in CB_2R-expressing cells (p = 0.0647 vs basal). Phosphorylated STAT3 was significantly reduced (p < 0.01 vs basal) regardless of CB_2R status following ConA treatment. Finally, both PTP1B and TC-PTP were upregulated in response to the T cell mitogen in $CB_2R^{+/+}$ (p < 0.05 and p = 0.0605 vs basal, respectively) and $CB_2R^{-/-}$ (p = 0.0831 and p < 0.001 vs basal, respectively) immune cells.

When examining the effects of cannabinoids on activated immune cells, neither HU-308 nor THC altered the total expression of STAT1 and STAT3 in either genotype. However, both compounds significantly decreased STAT3 phosphorylation in CB_2R-expressing cells (p < 0.05 vs ConA-activated), without significantly affecting pSTAT1 levels in this population. In CB_2R-lacking cells, HU-308 did not modify STAT1 or STAT3 phosphorylation, whereas THC reduced both (p < 0.05 and p = 0.0714 vs ConA activated, respectively). Regarding negative regulators, PTP1B was increased by cannabinoid treatment in $CB_2R^{+/+}$ immune cells (p < 0.05 vs ConA-activated), while TC-PTP remained unchanged. Unexpectedly, while neither agonist modifies PTP1B levels in $CB_2R^{-/-}$ immune cells, HU-308 significantly increased TC-PTP expression in this population (p < 0.05 vs ConA-activated).

Although mechanistically complex, these findings suggest that cannabinoids impair JAK/STAT signalling by a yet unidentified target distinct from CB_2R. Further experiments will be required to understand the physiological significance and the mechanisms underlying these effects.

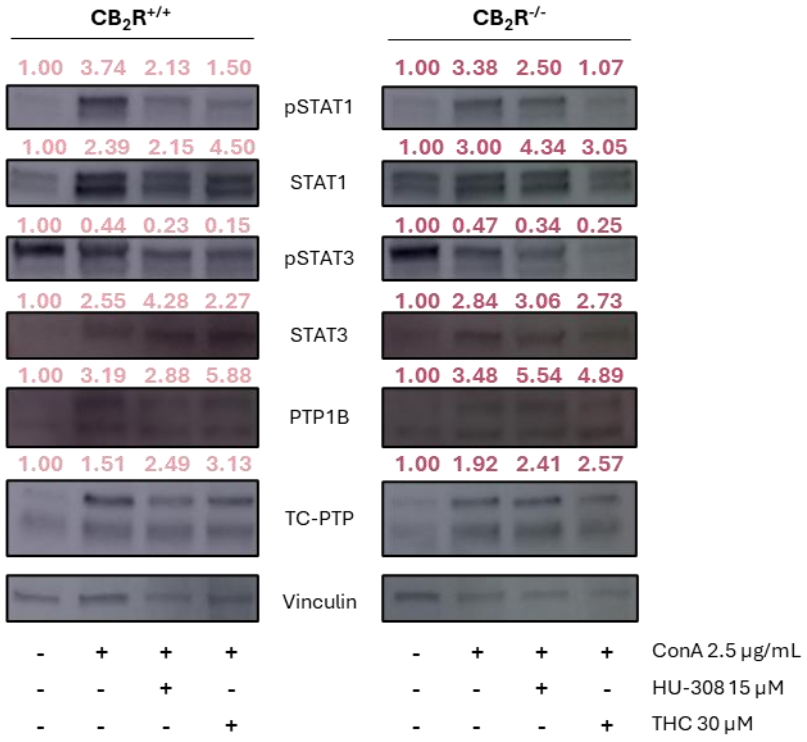

Figure R70. Analysis of the JAK/STAT cascade in splenocytes obtained from the C57BL/6 model. Representative Western blots of the indicated JAK/STAT signalling pathway elements and the negative regulator phosphatases in cultures of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) female mice activated for 24 h with ConA and then treated with the indicated cannabinoid for 6 h. Densitometric values were first normalised to Vinculin or total protein and then to the non-treated group, that was set as 1. The mean value of 3 independent experiments is shown above each membrane slice. Student's t-test vs vehicle-treated or ConA-activated.

Together, these results suggest that cannabinoids may exert immunosuppressive-like effects through CB_2R-independent mechanisms in activated immune cells. These effects appear to be primarily mediated by the regulation of cell survival and intracellular signalling—as inferred from the reduction in immune cell viability and alterations in the JAK/STAT cascade—, rather than by interference with cell activation or effector function as PD-1 expression and IFN-γ production remained unaffected.

The immunosuppressive effect of cannabinoids on activated immune cells was confirmed through co-culture with AT3 BC cells (**Figure R71**). Treatment with both HU-308 and THC significantly reduced the ability of both $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes to eliminate tumour cells. However, this effect was less pronounced in CB_2R-lacking immune cells, with significant differences at the highest concentrations of cannabinoid tested. These results point to a partial contribution of CB_2R to the reduction in splenocyte killing capacity, but, consistent with previous models, indicate that cannabinoid action on immune cells is largely mediated by other yet unidentified targets.

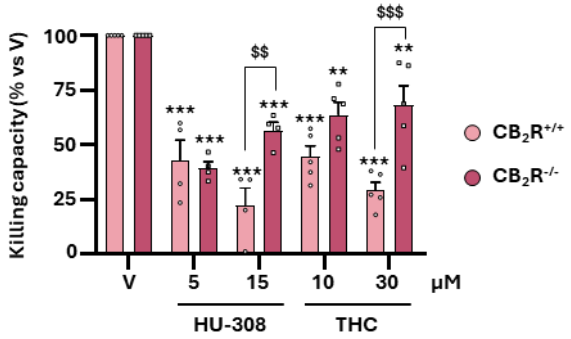

Figure R71. Analysis of the killing capacity of splenocytes obtained from the C57BL/6 model. The killing capacity of C57BL/6 $CB_2R^{+/+}$ (light pink) and $CB_2R^{-/-}$ (dark pink) splenocytes activated for 24 h with ConA and then cultured in the presence of cannabinoids for another 24 h was assessed in co-cultures of these cells with AT3 cancer cells (ratio E:T 50:1) and determined as 100 minus the viability (%) of AT3 cells. These values were then relativised to that of the vehicle-treated (V) group, that was set as 100. Two-way ANOVA: ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells; $$ $p < 0.01$, $$$ $p < 0.001$ vs $CB_2R^{+/+}$.

Effects on splenocytes isolated from the CB_2R^{eGFP} model

To further study the impact of cannabinoid treatment after activation, we conducted flow cytometry experiments using splenocytes isolated from C57BL/6 CB_2R^{eGFP} females.

As in the previous experimental model, the overall reduction in immune cell viability (**Figure R72A**), was primarily attributable to decreased viability of GFP^+ (CB_2R^+) cells, whereas GFP^- (CB_2R^-) cells were not affected (**Figure R72B**).

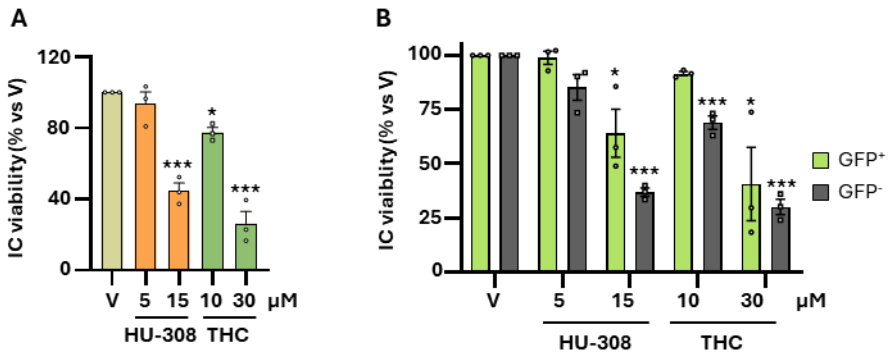

Figure R72. Effect of cannabinoids on the viability of splenocytes obtained from the CB_2R^{eGFP} model. Overall immune cell viability (A) or stratified according to GFP (CB_2R) expression (B) of splenocytes isolated from CB_2R^{eGFP} female mice and activated for 24 h with ConA followed by culture in the presence of cannabinoids for another 24 h. Viability, assessed by flow cytometry, was relativised to that of the vehicle-treated (V) group, that was set as 100. One-way ANOVA: * $p < 0.05$, *** $p < 0.001$ vs vehicle-treated cells.

Flow cytometry analysis revealed that PD-1 expression remained unchanged in response to HU-308 treatment, whereas THC induced an overall increase in PD-1 within the total immune population (**Figure R73A**). This effect was not attributable to T cells, as PD-1 levels in this subset decreased in response to the highest concentration of THC (**Figure R73C**), suggesting that other immune cell types should be responsible for the overall phenotype. Upon differentiation between GFP^+ (CB_2R^+) and GFP^- (CB_2R^-) cells, THC significantly upregulated PD-1 in CB_2R-expressing immune cells but downregulated it in CB_2R-lacking cells (**Figure R73B**). Only changes in CB_2R-lacking T cells could be the responsible for the overall effect observed in this population, whereas other CB_2R-expressing cell types likely account for to the overall PD-1 upregulation (**Figure R73D**).

These results indicate that cannabinoids reduce immune cell viability of ConA-stimulated immune cells independently of CB_2R expression, but do not substantially modify their activated phenotype.

Collectively, our results from both the C57BL/6 and the CB_2R^{eGFP} models indicate that cannabinoids exert immunosuppressive-like effect regardless of CB_2R expression. These mechanisms, which operate across all stages of immunosurveillance, a characterised by impaired cell survival and dysregulated intracellular signalling. In cells undergoing activation, cannabinoids additionally disrupt both activation processes and effector functions, including the release of antitumour cytokines.

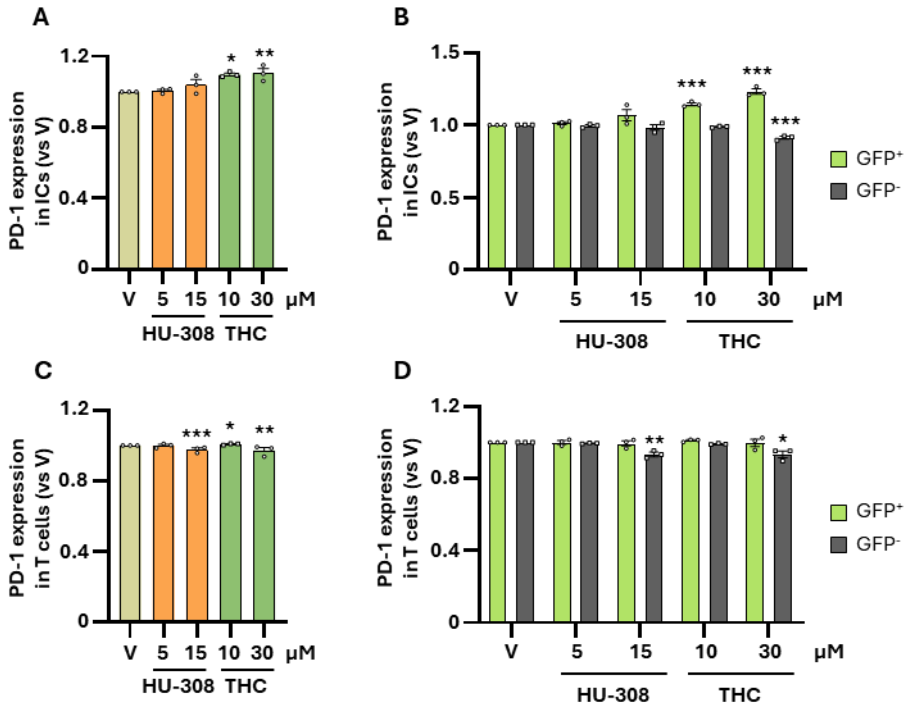

Figure R73. Effect of cannabinoids on the activation marker PD-1. Overall PD-1 expression (A and C) or stratified according to GFP (CB_2R) expression (B and D) in total immune cells (A-B) or T cells (C-D) isolated from CB_2R^{eGFP} female mice and activated for 24 h with ConA followed by culture in the presence of cannabinoids for another 24 h. PD-1 expression, assessed by flow cytometry, was relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

EFFECT OF CANNABINOIDS ON IMMUNE AND CANCER CELL CO-CULTURES

Finally, to better recapitulate the complex functional interactions between immune and cancer cells within the tumour microenvironment, we evaluated the consequences of cannabinoid treatment in a co-culture system. Unlike the previous models, where only immune cells were directly exposed to cannabinoids, here, co-cultures of ConA-activated splenocytes and cancer cells were simultaneously challenged. Specifically, ConA-stimulated splenocytes were co-cultured with BC cells for 24 h prior to the addition of cannabinoids for an additional 48 h, as illustrated in **Figure R74.** This approach represents a more accurate model resembling cancer patients who receive (or consume) cannabinoids.

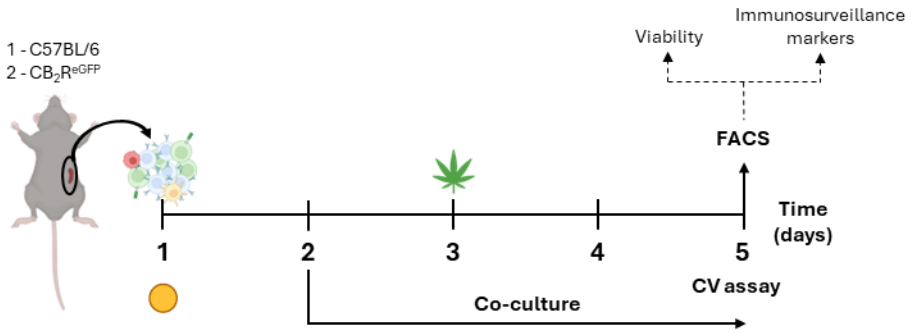

Figure R74. Experimental set up. Schematic representation of the strategy used to assess the effect of cannabinoids in a context where immune and cancer cells are interacting. Isolated splenocytes were activated for 24 h with ConA (yellow sphere icon) prior to their co-culture with AT3 BC cells. After 24 h of co-culture, cannabinoids (marijuana leaf icon) were added and incubation continued for an additional 48 h. Subsequently, flow cytometry (FACS) or crystal violet (CV) assays were performed.

First, we analysed the impact of cannabinoids on the viability of cancer cells. Our results confirmed a concentration-dependent reduction in AT3 cell viability in response to cannabinoid treatment when cancer cells were cultured alone (**Figure R75 and R76**).

Notably, co-culture with activated C57BL/6-derived $CB_2R^{+/+}$ and $CB_2R^{-/-}$ splenocytes (**Figure R75**) led to a significant reduction in cancer cell viability even in the absence of cannabinoids, with comparable effects irrespective of CB_2R status. Cannabinoid treatment in co-cultures also resulted in a significant concentration-dependent decrease in BC cell viability, that was higher than the one observed for cancer cells cultured alone at the lowest, but not at the highest, concentrations tested and independent of CB_2R expression.

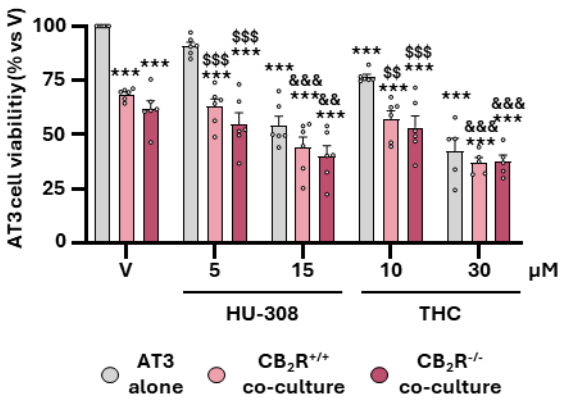

Figure R75. Effect of cannabinoid treatment on the viability of AT3 BC cells co-cultured with splenocytes obtained from the C57BL/6 model. Viability of AT3 BC cells, as assessed by crystal violet assay, cultured alone (grey bars) or in co-culture (ratio E:T 50:1) with C57BL/6 $CB_2R^{+/+}$ (light pink bars) and $CB_2R^{-/-}$ (dark pink bars) splenocytes and treated with cannabinoids or the corresponding vehicle (V). Two-way ANOVA: *** $p < 0.001$ vs vehicle-treated AT3 cells; $$ $p < 0.01$, $$$ $p < 0.001$ vs cannabinoid-treated AT3 cells; && $p < 0.01$, &&& $p < 0.001$ vs vehicle-treated co-culture.

To determine the specific contribution of CB_2R expressed by T cells, we performed analogous co-culture experiments using splenocytes from CB_2R^{eGFP} and CD4-$CB_2R^{-/-}$ female mice (**Figure R76**). The crystal violet assay yielded results virtually identical to those described above: co-culture with activated splenocytes resulted in a significant reduction in AT3 cell viability, and this effect was further enhanced by cannabinoids at the highest—but not at the lowest—concentrations tested, independently of CB_2R expression in T cells.

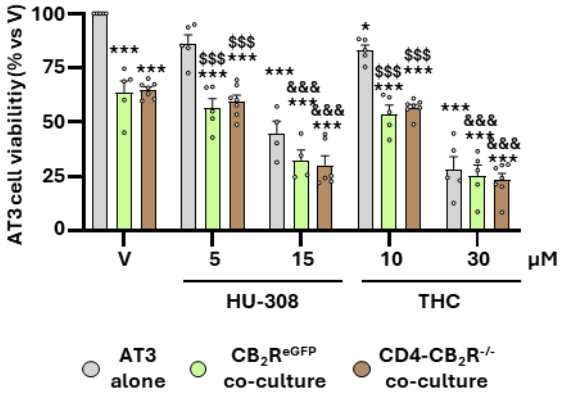

Figure R76. Effect of cannabinoid treatment on the viability of AT3 BC cells co-cultured with splenocytes obtained from the CB_2R^{eGFP} and CD4-$CB_2R^{-/-}$ models. Viability of AT3 BC cells, as assessed by crystal violet assay, cultured alone (grey bars) or in co-culture (ratio E:T 50:1) with CB_2R^{eGFP} (green bars) and CD4-$CB_2R^{-/-}$ (brown bars) splenocytes and treated with cannabinoids or the corresponding vehicle (V). Two-way ANOVA: * $p < 0.05$, *** $p < 0.001$ vs vehicle-treated AT3 cells; $$$ $p < 0.001$ vs cannabinoid-treated AT3 cells; &&& $p < 0.001$ vs vehicle-treated co-culture.

These results suggest that CB_2R expression in T cells alone is not enough to limit their cytotoxic capacity, highlighting the importance of the microenvironment in shaping antitumour immune responses. Moreover, they suggest that cannabinoids exert potent antitumoural effects but may simultaneously limit immune cell contribution to cancer cell killing, as the combined effect of cannabinoids and immune cells was only marginally greater than that of either component alone. Importantly, these effects occurred independently of CB_2R expression both in the total immune cell pool and specifically within T cells.

We then analysed the contribution of immune cells to cancer cell death in the co-cultures following cannabinoid treatment. Even though we observed increased cancer cell death in the presence of immune cells (what we interpreted as a sign of immune cell activation), our results showed that immune cell viability was compromised by cannabinoids in a concentration-dependent manner (**Figure R77**).

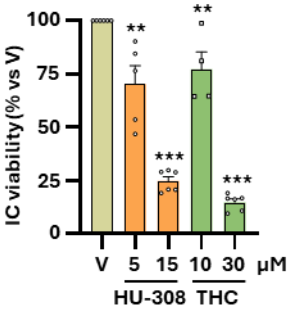

Figure R77. Effects of cannabinoids on the viability of splenocytes co-cultured with AT3 cells. Viability, as assessed by flow cytometry, of splenocytes isolated from C57BL/6 $CB_2R^{+/+}$ female mice and co-cultured with AT3 BC cells in the presence of cannabinoids. Viability was relativised to that of the vehicle-treated (V) group, that was set as 100. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

Moreover, cannabinoid treatment significantly increased total PD-1 expression (**Figure R78**). We hypothesise that this effect may reflect immune cell exhaustion rather than early activation. Further characterisation of the specific PD-1 expressing populations—particularly cytotoxic T cells—will be necessary to clarify the dynamics of PD-1 regulation following cannabinoid treatment, as well as its functional relevance and the potential role of CB_2R.

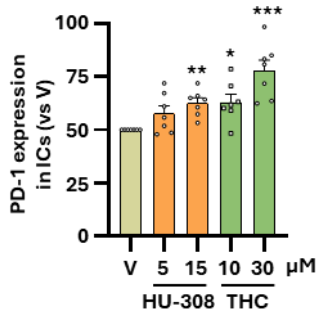

Figure R78. Effects of cannabinoids on the activation marker PD-1 in splenocytes co-cultured with AT3 cells. Expression of PD-1, as assessed by flow cytometry, in total immune cells isolated from C57BL/6 $CB_2R^{+/+}$ female mice and co-cultured with AT3 BC cells in the presence of cannabinoids. PD-1 expression was relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

Regarding BC cells, none of the cannabinoids significantly affected the expression of MHC class I (MHC-I) molecules (**Figure R79A**), indicating that their visibility to immune cells was neither enhanced nor diminished. In contrast, both HU-308 and THC significantly increased PD-L1 expression (**Figure R79B**), which would be expected to promote cytotoxic T cell anergy upon target recognition.

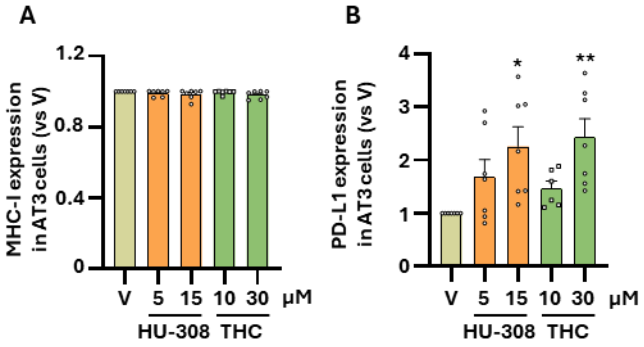

Figure R79. Effects of cannabinoids on immune evasion markers of AT3 cells co-cultured with splenocytes. Expression of MHC-I (A) and PD-L1 (B), as assessed by flow cytometry, in AT3 cells co-cultured with C57BL/6 $CB_2R^{+/+}$ immune cells in the presence of cannabinoids. MHC-I and PD-L1 expression were relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

Interestingly, analysis of AT3 cells cultured alone revealed an increase in MHC-I expression in response to THC (**Figure R80A**), as well as an increase in PD-L1 expression in response to both HU-308 and THC (**Figure R80B**). These results suggest that the presence of immune cells may constrain the cannabinoid-induced upregulation of MHC-I.

Taken together, our results suggest that, although cannabinoids may trigger immunosuppressive responses in splenocytes—regardless of timing of exposure and CB_2R expression—their antitumour effect on cancer cells predominates in a context where tumour-immune cell interactions occur (as in actual tumours). This reinforces the potential of cannabinoids as effective and safe antitumour agents.

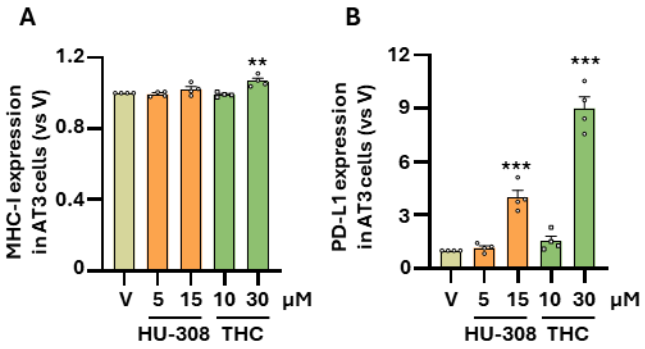

Figure R80. Effects of cannabinoids on immune evasion markers of AT3 cells cultured alone. Expression of MHC-I (A) and PD-L1 (B), as assessed by flow cytometry, in AT3 cells treated with cannabinoids. MHC-I and PD-L1 expression were relativised to that of the vehicle-treated (V) group, that was set as 1. One-way ANOVA: ** $p < 0.01$, *** $p < 0.001$ vs vehicle-treated cells.

DISCUSSION

BC, the leading cause of cancer-related death in women worldwide, is a highly heterogeneous disease in terms of molecular markers, patient prognosis and response to therapy. Despite significant advances in early diagnosis and treatments, there are still some challenges to overcome—including innate or acquired therapy resistance, the absence of targeted therapies for TNBC, or the incurability of metastatic disease—that underscore the ongoing needed for research in this field (Harbeck et al., 2019). Over the past few decades, the concept of cancer has evolved and is no longer considered a group of transformed malignant cells that proliferate uncontrollably, but rather a complex and heterogeneous ecosystem where tumour cells interact with their surroundings to receive signals that influence their proliferation, survival and metastatic potential. This TME, consisting of both non-cellular and cellular components, is now recognised as a key player in tumour progression and, more importantly, a promising source of both potential biomarkers and novel therapeutic targets (De Visser & Joyce, 2023). Understanding the dynamic crosstalk between cancer cells and the stroma may yield novel insights into mechanisms underlying disease initiation and progression.

In this context, the ECS has been found to be deregulated in numerous types of cancer, including BC (Moreno et al., 2019). Several studies have investigated how this system influences cancer cell fate both *in vitro* and *in vivo* (Velasco et al., 2016); however, its specific role within the TME remains largely unexplored. Given its high expression in the immune system – one of the main components of the TME – and its well-established immunomodulatory role, we decided to focus our attention on CB_2R. Notably, CB_2R is considered the primary target for the antitumoural effects of cannabinoids in preclinical models of BC (Blasco-Benito et al., 2019; Caffarel et al., 2006, 2010); however, activation of this receptor in immune cells has also been shown to exert immunosuppressive effects (Lombard et al., 2007; Massi et al., 2000; M. Yuan et al., 2002), underscoring the complexity of its influence within the TME. In this scenario, our aim was to elucidate the role of CB_2R expressed by immune cells in the TME in BC initiation and progression, and to assess the consequences of its pharmacological targeting in both immune and cancer cells.

CHARACTERISATION OF CB_2R EXPRESSION IN IMMUNE CELLS

We started this project by making an in-depth characterisation of CB_2R expression in different immune cell populations, including those residing in the BTME. Our results from the analysis of non-transformed tissue showed an enriched expression pattern in lymphoid tissues, consistent with previous reports (Galiègue et al., 1995; Turcotte et al., 2016). However, the analysis of isolated immune cell subpopulations, primarily of murine origin, revealed results that differ from those reported by others at both the mRNA and protein levels. Both *in silico* and *in vivo* (by using the CB_2R^{eGFP} mouse model), we found that cytotoxic T cells express high levels of CB_2R, nearly as high as those in B cells, whereas it had previously been reported that CB_2R expression in this population was almost negligible (Coopman et al., 2007; Galiègue et al., 1995; Graham et al., 2010; S. F. Lee et al., 2001a). The discrepancies between our study and others may be due to methodological differences such as the source of immune cells, purification methods, or the techniques employed to detect the *Cnr2* transcript [for example, the use of qPCR and specific primer sets, given that two isoforms for human and mouse CB_2R have been reported (Liu et al., 2009), vs high-throughput technologies such as RNA-seq] and the CB_2R protein (for example, the use of

antibodies with limited reliability vs reporter protein strategies). Nonetheless, our results are supported by Xiong and Yuan findings who, using a CB_2R^{eGFP} reporter mouse model like ours, also detected high levels of CB_2R in naive CD8 T cells isolated from murine spleens (X. Xiong et al., 2022; C. Y. Yuan et al., 2021). Furthermore, a model based on GFP as a reporter for CB_2R expression allowed Castaneda and colleagues to demonstrate that the receptor is not only present at the plasma membrane but also localised intracellularly. This observation enabled them to describe that, in T cells, CB_2R is predominantly found in the intracellular compartment and that, overall, its expression in this population is similar to that of B cells at both mRNA and protein levels (Castaneda et al., 2013).

Our analysis of scRNA-seq data from the Human Protein Atlas (HPA) project (Uhlén et al., 2015) pointed also at CD8 T cells as one of the resident immune populations with the highest CB_2R expression in the non-transformed human mammary gland. This, together with their pivotal role in immune defence in the non-transformed breast (Jaquish et al., 2024) and in the antitumour immune response in breast tumour tissue (Amens et al., 2021), led us to further investigate CB_2R expression throughout the lifespan of this cell type. Our *in silico* findings showed that cytotoxic T cells, both human and murine, decrease CB_2R levels in response to an activating stimulus. Notably, various studies support the downregulation of CB_2R upon activation (McKallip, Lombard, Martin, et al., 2002; Schatz et al., 1997) or under inflammatory conditions (C. Y. Yuan et al., 2021), whereas the vast majority of scientific evidence suggests that the receptor is upregulated in activated T cells compared to resting ones or in T cells found in inflamed tissues (Coopman et al., 2007; Maddukuri et al., 2022; Maes et al., 2023; Robinson et al., 2013). The discrepancies between our results and those of other studies may be attributable to the methodological differences outlined above, as well as to variations in the nature, concentrations and duration of the stimulus. In our *in vivo* model, splenic cytotoxic T cells isolated from tumour-bearing female mice (with the presence of tumour cells acting as the activating stimulus) exhibited significantly lower levels of CB_2R compared to those from non-tumour bearing animals. This supports the idea that CB_2R is downregulated in activated cells, although further functional analyses comparing non-tumour and tumour-bearing female-derived CD8 T cells would be required to confirm whether the latter are indeed activated.

In immune cells, we found that CB_2R expression does not significantly change with tumour progression but does differ from to the basal state (i.e., in immune cells from non-tumour bearing mice). Specifically, tumour-infiltrating B cells express significantly lower CB_2R levels than their naive counterparts. Indeed, it has been described that CB_2R is downregulated during B cell differentiation and activation (Carayon et al., 1998; Castaneda et al., 2017; S. F. Lee et al., 2001b). Based on this evidence, we hypothesise that B cells within the BTME may be more differentiated or activated, although further experiments will be necessary to confirm the specific functional status of these cells.

Tumour-infiltrating CD8 T cells also exhibited reduced CB_2R expression compared to the non-tumour population. In small tumours, we hypothesise that this may indicate recent activation, a notion supported by findings from Yuan and colleagues, who reported a downregulation of CB_2R in activated cytotoxic T cells in a model of graft-versus-host disease (C. Y. Yuan et al., 2021). However, in large tumours, low CB_2R expression is more likely to be associated with exhaustion rather than with a functional response. Indeed, our analysis showed higher and more sustained PD-1 expression in large tumours compared to small ones, which has been associated with cytotoxic T cell dysfunction (Jenkins et al., 2023).

Analysis of other inhibitory receptors implicated in exhaustion [such as TIM-3 or LAG-3 (Jenkins et al., 2023)] could help to clarify the phenotype of this population at different stages of tumour progression. Additionally, it would be of interest to assess CB_2R expression in cytotoxic T cells isolated from the tumour-draining lymph node—where the antitumoural immune response primarily starts (Okamura et al., 2022)—and compare this with CB_2R levels in cells from baseline (non-tumour-influenced) lymph nodes. This analysis would help elucidate the regulatory mechanisms governing CB_2R expression and clarify whether tumour-infiltrating CD8 T cells already exhibit reduced CB_2R levels prior to entering the BTME, or if CB_2R downregulation is completed within the tumour itself.

As their cytotoxic counterparts, helper T cells exhibited diminished CB_2R expression within the BTME compared to their naive state. Again, Yuan and collaborators reported CB_2R downregulation in CD4 T cells from graft-versus-host disease compared to naive counterparts (C. Y. Yuan et al., 2021), suggesting that this could be indicative of strong T cell activation. Based on this evidence, we hypothesise that CB_2R may limit CD4 T cell activation, although future experiments will be needed to confirm this hypothesis and to further identify the specific(s) subsets of helper T cells infiltrating the tumours (e.g., Th1, Th2, Treg, etc.) and their relative CB_2R levels.

In contrast to B and T cells, CB_2R levels in tumour-infiltrating myeloid cells were higher than those in naive cells. It has been reported that in models where inflammation, a hallmark of cancer (Hanahan, 2022), is a predominant feature—such as traumatic brain injury (Braun et al., 2018) or neuropathic pain (Nent et al., 2019)—there is a dramatic upregulation of CB_2R within infiltrating myeloid cells. Future experiments will be necessary to determine whether this upregulation translates into a more anti-inflammatory and, consequently, pro-tumoural phenotype. In NK cells, we also observed upregulation of CB_2R compared to the basal state but found no evidence of CB_2R expression modulation in response to any stimulus in this population. Notably, although it has been widely stated that NK cells express high levels of CB_2R (Galiègue et al., 1995; Graham et al., 2010), our study revealed that expression is not as high as previously thought, which could be due to the lack of distinction between NK and NKT cells in previous studies. Further experiments will be required to elucidate the functional consequences of CB_2R upregulation in tumour-infiltrating NK cells.

Our study also provides new data on the role of CB_2R in regulating the expression of immunosurveillance markers, particularly in a context where cells expressing and lacking the receptor coexist. We found that $CB_2R^{-/-}$ cytotoxic T cells exhibit higher PD-1 levels in small tumours compared to their WT counterparts, suggesting that CB_2R raises the activation threshold required for CD8 T cell activation (i.e., limits their hyperactivation) during the early stages of the antitumoural immune response. This hypothesis is further supported by the lower PTP1B expression observed in splenocytes isolated from non-tumour bearing $CB_2R^{-/-}$ mice compared to their WT counterparts, as PTP1B acts to limit T cell activation (S. Liang et al., 2023). In the literature, information regarding PD-1 expression in CB_2R-lacking cytotoxic T cells is contradictory. On the one hand, Xiong and colleagues observed a decrease in PD-1 levels in T cells from tumours of $CB_2R^{-/-}$ mice compared to $CB_2R^{+/+}$ mice in a melanoma model (X. Xiong et al., 2022). On the other hand, the Schicho's group described an increase in PD-1 in CB_2R-lacking CD8 T cells compared to their WT counterparts in an NSCLC model (Sarsembayeva et al., 2023). It is important to note that both studies were conducted in different tumour models, and the analyses were performed

at distinct tumour sizes. In any case, our results are compatible with previous observations and provide a more comprehensive perspective on the influence of CB_2R in regulating PD-1. Additional studies using cytotoxic T cells directly isolated from the BTME at various stages of tumour progression would help clarify whether $CB_2R^{-/-}$ cells are indeed more activated in small tumours than their WT counterparts, thereby providing a more comprehensive picture of the role of CB_2R in antitumour immunity.

Unlike PD-1, PD-L1 was virtually undetectable in CB_2R-lacking myeloid and helper T cells, regardless of tumour size. This results sharply contrast with the observations of Sarsembayeva and collaborators, who detected enhanced expression of PD-L1 on myeloid cells of $CB_2R^{-/-}$ mice compared to their $CB_2R^{+/+}$ counterparts (Sarsembayeva et al., 2023). This discrepancy may be attributable to differences in the tumour model, highlighting the importance of the tumour context when interpreting the results. It is well established that one of the driving factors for PD-L1 expression in cancer cells is the binding of IFN-γ to its heteromeric receptor and the subsequent activation of the JAK/STAT signalling pathway; this mechanism is also relevant in myeloid cells (J. Qian et al., 2018). By Western blot, we observed that CB_2R-lacking immune cells express lower levels of IFNGR1—the major ligand-binding subunit of the receptor, (Tau & Rothman, 1999)—than their WT counterparts. In the presence of CB_2R-expressing cells (as in the CB_2R^{eGFP} model), the reduced bioavailability of IFNGR1 in $CB_2R^{-/-}$ immune cells could compromise their ability to respond to IFN-γ, resulting in lower PD-L1 expression. Further experiments will be required to determine whether the observed differences in the IFN-γ/IFNGR signalling pathway support our hypothesis and whether this mechanism applies to both myeloid and CD4 T cells.

Although our results remain largely preliminary, they also suggest that CB_2R may modulate the expression of CD69 and CD107α. Specifically, we have observed that the total pool of CB_2R-lacking immune cells displayed higher expression of both markers compared to CB_2R-expressing counterparts in T2 tumours, while the differences disappeared in larger tumours. These results do not appear to be attributable to variations in the levels of CD69 and CD107α within the immune subpopulations analysed—namely T, NK and NKT cells—when stratified according to CB_2R expression. However, it has been previously reported that $CB_2R^{-/-}$ T cells express higher levels of CD69 compared to their $CB_2R^{+/+}$ counterparts (Karmaus et al., 2012), and that $CB_2R^{-/-}$ NK cells exhibit elevated CD107α relative to $CB_2R^{+/+}$ NK cells (Sarsembayeva et al., 2023). To further elucidate the role of CB_2R in regulating immune cell activation and cytotoxicity within the BTME, it would be necessary to increase the sample size and evaluate additional cell populations that may also express CD69 and CD107α, such as polymorphonuclear cells, which are now beginning to be investigated in the context of BC (Garai et al., 2021; Obeagu & Obeagu, 2024). For instance, CD69 expression in human neutrophils is induced by various cytokines (Atzeni et al., 2002), some of which—such as GM-CSF (Guo et al., 2025) or IFN-γ (Yi et al., 2024)—are present within the TME. Furthermore, CD107α is increased in activated compared to resting basophils (Hennersdorf et al., 2005). Although direct analysis of CD107α expression was not assessed, cytokines present within the BTME such as IL-33 (Stojanovic et al., 2023) have been shown to upregulate granzyme B (a component of cytotoxic granules that also contain CD107α) mRNA and surface expression of the degranulation marker CD63 in this cell population (Marone et al., 2020).

Collectively, our results show that CB_2R is expressed by immune cells within the BTME, with expression levels distinct from those observed in their naive counterparts. Moreover, they

suggest that CB_2R regulates antitumour immunity by acting as a negative regulator of CD8 T cell activation in early stages of carcinogenesis (i.e., by limiting PD-1 expression) and by promoting PD-L1-mediated immunosuppression by other immune cell types.

ANALYSIS OF THE ROLE OF BTME-CB_2R IN THE INITIATION AND PROGRESSION OF BC

Several studies have reported alterations in CB_2R expression in BC compared to non-transformed tissue, albeit with contradictory results. On the one hand, our group has demonstrated that CB_2R transcripts and protein are scarcely detectable in the non-transformed mammary gland, whereas its expression is elevated across all BC subtypes, and correlates with poorer outcomes (Caffarel et al., 2006, 2010; Pérez-Gómez et al., 2015). On the other hand, Song and collaborators reported that CB_2R expression is lower in cancerous compared to adjacent tumour tissue, and that high levels are associated with a more favourable prognosis (Q. Song et al., 2023). It is important to mention that these studies compared bulk transformed and non-transformed breast tissue, potentially masking important information regarding the individual contributions of the epithelium and the stroma. Although we have not performed an in-depth dissection of BTME populations, we found that CB_2R is upregulated in the tumour stroma compared to the non-transformed stroma, and that this overexpression correlates with an increased risk of relapse, specifically in patients with TNB tumours.

The evidence regarding the function of CB_2R in the tumour stroma is limited and occasionally contradictory, with reported effects varying according to the tumour model studied. For example, in murine models of melanoma (Gruber et al., 2021) or hepatocarcinoma (Suk et al., 2016), an antitumour effect mediated by TME-CB_2R has been described. On the contrary, studies in melanoma, colon cancer and Lewis lung adenocarcinoma (X. Xiong et al., 2022) as well as NSCLC (Sarsembayeva et al., 2023) have found a pro-tumoural effect of TME-CB_2R, suggesting that the role of this receptor may be tumour-dependent. In the specific case of BC, the evidence is even more limited and arises primarily from our own group. Using a mouse model of metastatic HER2-enriched BC with global deletion of CB_2R (i.e., in tumour and non-tumour cells), Pérez-Gómez and colleagues reported a pro-tumoural role of CB_2R (Pérez-Gómez et al., 2015). Among other things, they observed that $CB_2R^{-/-}$ animals exhibited reduced tumour incidence and growth and fewer metastatic lesions compared to their WT counterparts. Nonetheless, this model does not allow to determine the relative contribution of the CB_2R expressed by the tumour cells and that expressed by the stroma—a question that constitutes the main objective of our current study. Here, we have observed that CB_2R expression within the TME does not significantly affect tumour initiation or progression, nor the composition of the BTME—including immune cell infiltration and soluble factor profiles—in luminal B and HER2-enriched BC subtypes. In contrast, it exerts a pro-tumoural and pro-metastatic role in TNBC, altering the immune composition and soluble component of the primary tumour and favouring colonisation of secondary organs by cancer cells. These differences between subtypes may be due to the higher immunogenicity and greater immune cell content in TNB tumours compared to Luminal B and HER2-enriched tumours (Abdou et al., 2022), which could make any alterations in immune regulatory mechanisms—such as those mediated by CB_2R—more relevant in this specific subtype. However, we cannot rule out that the intrinsic

properties of tumour cells themselves could dictate how they respond to CB_2R-mediated signals from the TME, potentially explaining the different results obtained in the three BC subtypes. To further elucidate if the observed results depend on CB_2R expression in the stroma, in the cancer cells or on the interaction between both, experiments could be performed using $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumour cells orthotopically implanted into $CB_2R^{+/+}$ and $CB_2R^{-/-}$ animals.

In our model of TNBC, we observed that small tumours lacking CB_2R tended to exhibit a higher percentage of NK cells compared to CB_2R-expressing tumours. This finding is noteworthy given the role of this population as early effectors in antitumour immunity and their association with better outcomes in BC patients (Nersesian et al., 2021). While the hypoxic TME is known to impair NK function—leading to reduced activation and a shift towards a non-cytotoxic phenotype (Barsoum et al., 2011; Thacker et al., 2023)—our data indicate that, although the percentage differences in NK cells diminish as tumour progress, CB_2R-lacking NK cells consistently express higher levels of CD69 and CD107α compared to their CB_2R-expressing counterparts. This observation suggests that, despite the general decline in NK function during tumour progression, $CB_2R^{-/-}$ NK cells may retain a greater functional activity and continue to participate in tumour cell elimination in larger tumours. This interpretation is further supported by previous studies showing higher CD107α expression after *ex vivo* stimulation in NSCLC models lacking stromal CB_2R (Sarsembayeva et al., 2023); as well as enhanced IFN-γ production by $CB_2R^{-/-}$ NK cells in a model of airway inflammation (Ferrini et al., 2017). It is important to note that our findings in this model differ from those observations in the CB_2R^{eGFP} mouse model, but as mentioned in the corresponding Results section, the small sample size in the latter warrants cautious interpretation. Nonetheless, our results suggest that CB_2R may favour the dysfunction associated to tumour progression of NK cells, although further experiments will be needed to more deeply assess the role of CB_2R in the activity of this tumour-infiltrating population.

We also observed quantitative differences in the myeloid infiltrate between $CB_2R^{-/-}$ and $CB_2R^{+/+}$ tumours. Myeloid cells represent one of the major populations of tumour-infiltrating immune cells in BC, with TAMs and MDSCs being the most abundant subsets (Cha & Koo, 2020). TAMs are known for their functional heterogeneity, with M1-TAMs—mainly pro-inflammatory and antitumoural—predominating in the earlier stages of carcinogenesis, whereas M2-TAMs—considered anti-inflammatory and pro-tumoural—becoming more prevalent in intermediate and late stages (Khan et al., 2023). While both genotypes may contain M1-TAMs in early stages, our findings suggest that CB_2R-lacking tumours display a greater initial infiltration of myeloid cells with characteristics consistent with a pro-inflammatory phenotype. This is supported by evidence from a *Salmonella* infection model, where CB_2R deficiency in macrophages results in a pronounced M1 profile (Barker et al., 2025), as well as by a study where $CB_2R^{-/-}$ mice exhibited lower levels of immunosuppressive $CXCR3^{hi}$ macrophages in the gut (Acharya et al., 2017). Furthermore, highly activated myeloid cells—those displaying maximal effector functions such as antigen presentation, phagocytosis, and cytokine release—have been reported to express low levels of CB_2R (Carlisle et al., 2002), further supporting the notion that $CB_2R^{-/-}$ myeloid cells may be more functionally active and antitumoural. In our CB_2R^{eGFP} mouse model, we observed that tumour-infiltrating myeloid cells expressed significantly higher levels of CB_2R compared to their counterparts isolated from the spleen of non-tumour bearing females. Based on the evidence presented here, we hypothesise that CB_2R expression in tumour-infiltrating myeloid cells—whether as a cause or a consequence of the BTME—acts to limit their pro-

inflammatory effector functions, thereby favouring tumour growth, although further experiments will be required to validate our hypothesis.

For the identification of myeloid cells by flow cytometry, we used the pan-marker CD11b. Interestingly, it has been reported that CD11b activation promotes M1-TAM polarization, whereas an immunosuppressive TME can downregulate its expression and favour M2-TAM polarization (M. C. Schmid et al., 2018). These findings suggest that the observed decrease in the proportion of CD11b cells as the tumour progressed—also observed in the CB_2R^{eGFP} mouse model—may not necessarily reflect reduced myeloid cell infiltration, but rather a shift towards a more immunosuppressive phenotype—consistent with the reported predominance of M2-TAMs in later stages of carcinogenesis. This transition may be delayed in the absence of CB_2R, as suggested by the slower activation of remodelling M2-TAMs observed in a model of ischemic cardiomyopathy (Duerr et al., 2014). Although substantial evidence suggests that CB_2R deficiency in TAMs results in a pro-inflammatory and antitumoural phenotype, we cannot exclude the contribution of the other myeloid subsets within the TIME (i.e., MDSC). Indeed, lack of CB_2R has been associated with a trend toward higher MDSC infiltration in models of non-melanoma skin carcinogenesis (Iden, Raphael-Mizrahi, Naim, et al., 2023) or enhanced splenic populations of MDSCs in colon cancer models (Iden, Raphael-Mizrahi, Awida, et al., 2023). However, it is important to note that these results were obtained in models of spontaneous or chemically induced carcinogenesis, in which tumour cells also lacked CB_2R in knockout animals, thereby precluding the isolated analysis of stromal CB_2R contribution to the observed outcome. Nevertheless, further experiments will be needed to determine the specific myeloid cell subtypes infiltrating the BTIME, the functional consequences of CB_2R deletion in each subset and, therefore, the role of this receptor in each myeloid subpopulation.

In larger TNBC tumours, we observed a greater infiltration of B cells in CB_2R-lacking tumours compared to their CB_2R-expressing counterparts, a finding that is clinically relevant since increased tumour-infiltrating B cells is generally associated with improved prognosis in BC patients (M. Li et al., 2023). Modulation of CB_2R expression has demonstrated to be essential for the formation and regulation of specific B cell subsets. For example, CB_2R is required for the retention of B cells within bone marrow sinusoids, with its absence leading to increase in blood immature B cells (Pereira et al., 2009). This may have direct implications for tumour infiltration, as Gruber and colleagues found that lack of stromal CB_2R in a mouse model of melanoma results in a higher infiltration of less differentiated B cells, potentially fostering an immunosuppressive microenvironment through the induction of regulatory T cells (Gruber et al., 2021). Moreover, CB_2R deficiency has been associated with a significant reduction in marginal zone B cells (Basu et al., 2011), as well as in splenic germinal centre and memory B cells (Basu et al., 2013). Despite these alterations, CB_2R does not seem to be required for general B cell development or function, as no significant changes in the total number of mature B cells (Pereira et al., 2009), in T-dependent antibody responses, or in germinal centre B cell formation (Basu et al., 2013) have been reported in its absence. Of note, germinal centre centroblasts—characterised by low expression of CB_2R (Carayon et al., 1998)—are major components of tertiary lymphoid structures, a feature associated with improved prognosis in BC (M. Li et al., 2023). Considering these evidences, currently we can only propose that CB_2R restrains B cell infiltration during tumour progression. Further experiments will be required to determine whether CB_2R deletion also leads to changes in tumour-infiltrating B cell phenotype and whether such

changes (if any) could help explain the differences in tumour growth observed between genotypes.

We also observed a greater infiltration of cytotoxic T cells in larger TNBC tumours lacking stromal CB_2R compared to those expressing the receptor, which is consistent with the fact that B cell levels are highly correlated with the density of tumour-infiltrating T cells (M. Li et al., 2023). Furthermore, increased T cell infiltration is generally associated with improved clinical outcomes in BC (Mahmoud et al., 2011; Oshi, Asaoka, et al., 2020; Sun et al., 2022; Triulzi et al., 2015). Our results are corroborated by studies in animal models of melanoma, colon cancer and Lewis lung adenocarcinoma (X. Xiong et al., 2022), as well as in NSCLC (Sarsembayeva et al., 2023), which have reported enhanced infiltration and cytotoxic activity of CD8 T cells in $CB_2R^{-/-}$ tumours. However, it is important to note that Gruber and colleagues observed a significant reduction in cytotoxic T cell frequencies in the absence of CB_2R in a melanoma model (Gruber et al., 2021). These contrasting findings indicate that the relationship between CB_2R expression and T cell infiltration may vary depending on the tumour type and the stage of tumour progression at the time of analysis. Specifically in our model of TNBC, our results suggest that CB_2R acts as a negative regulator of the infiltration of CD8 T cells in larger tumours, potentially facilitating immune evasion. The TME is known to promote cytotoxic T cell dysfunction, typically characterised by high expression of exhaustion markers such as PD-1 (Jenkins et al., 2023). In our C57BL/6 model, as well as in the CB_2R^{eGFP} mouse model, we did not observe differences in PD-1 levels between $CB_2R^{+/+}$ and $CB_2R^{-/-}$ tumours at advanced stages, suggesting a similar degree of T cell exhaustion irrespective of CB_2R status. This interpretation is further supported by our observations that CD69 levels were high and similar between the two genotypes at this tumour stage (a result also obtained in the CB_2R^{eGFP} mouse model). Notably, a transcriptomic analysis reported enhanced CD69 expression in pre-exhausted and exhausted CD8 T cells isolated from NSCLC, hepatocellular carcinoma and colorectal cancer (Beltra et al., 2020). Despite the general reduction in CD107α levels observed with tumour progression—consistent with the loss of granzyme B production at advanced stages of exhaustion (Jiang et al., 2015)—CB_2R-lacking T cells retained higher CD107α expression than their CB_2R-expressing counterparts. Taken together, the higher presence of CD107α in the cell surface alongside similar levels of CD69 and PD-1 in $CB_2R^{-/-}$ T cells could indicate a partially exhausted phenotype with residual effector function. These data suggest that CB_2R promotes the dysfunction associated with tumour progression of cytotoxic T cells. Nevertheless, additional experiments—such as direct *ex vivo* cytotoxicity assays—will be required to fully assess the role of CB_2R in the cytotoxic potential of tumour-infiltrating CD8 T cells.

Collectively, our data suggest that CB_2R plays a negative regulatory role in antitumour immunity by limiting the infiltration of immune cells that are generally associated with a favourable prognosis in BC and by promoting the tumour progression-associated dysfunction of cytotoxic cells. Given that the efficacy of immunotherapies targeting the PD-1/PD-L1 axis is highly dependent on the presence and functionality of effector immune cells within the TME, we hypothesise that CB_2R may limit susceptibility or response to these therapies. Indeed, in a model of NSCLC, $CB_2R^{-/-}$ mice showed improved responses to anti-PD-1 therapy compared to their $CB_2R^{+/+}$ counterparts (Sarsembayeva et al., 2023). An additional observation that supports our hypothesis is the fact that CB_2R-lacking immune cells express significantly lower basal levels of the phosphatase PTP1B, an intracellular T cell checkpoint that limits T cell expansion and cytotoxicity (S. Liang et al., 2023). Notably, *Ptpn1*$^{-/-}$ knockout mice display marked attenuation of tumour growth across several cancer

types, including BC, and exhibit increased abundance of TILs than their WT counterparts (Wiede et al., 2022), mirroring our findings in $CB_2R^{-/-}$ vs $CB_2R^{+/+}$ mice. Moreover, PTP1B inhibition has been shown to increase T cell-mediated antitumour immunity and supress tumour growth and can even sensitise therapy-resistant tumours to PD-1 blockade (S. Liang et al., 2023; Wiede et al., 2022). Future experiments will be needed to determine whether CB_2R-lacking TILs indeed express lower levels of PTP1B and/or the closely related phosphatase TC-PTP and the relative activity of both phosphatases. Furthermore, it will be interesting to assess if AT3 tumours—largely resistant to anti-PD-1 blockade—developed in $CB_2R^{-/-}$ mice are more sensitive to this immunotherapy compared to $CB_2R^{+/+}$ controls. With the aim of translating our results into the clinic, it would be interesting to investigate whether there is a correlation between CB_2R and PTP1B levels in the BTME, particularly in cytotoxic cells, in human BC patient samples. We could also study CB_2R levels before and after immunotherapy to determine whether CB_2R could be used as a predictive biomarker of response to this type of therapy. Indeed, our group has already described that CB_2R could be used as a prognostic biomarker in HER2-enriched BC (Pérez-Gómez et al., 2015) and as a predictive biomarker of response to anti-HER2 therapy (mainly trastuzumab) (Seijo et al., manuscript submitted).

Unlike in the CB_2R^{eGFP} model, where GFP^- (CB_2R^-) cells express virtually no PD-L1, in the C57BL/6 model, $CB_2R^{-/-}$ myeloid and helper T cells exhibit similar levels of PD-L1 as their $CB_2R^{+/+}$ counterparts. This suggests that CB_2R directly induces PD-L1 expression in immune cells, promoting a more immunosuppressive TME, but in its absence, extrinsic mechanisms controlled by the TME may upregulate PD-L1, possibly as a feedback mechanism to control intense immune activation. As mentioned in the previous section, one of the driving factors for PD-L1 expression is the activation of the IFN-γ/IFNGR signalling pathway. In principle, CB_2R-lacking immune cells might be expected to show reduced PD-L1 expression due to lower IFNGR1 expression. However, in the absence of CB_2R-expressing immune cells, CB_2R-lacking cells would be the sole responders to this cytokine, which could be enhanced in the $CB_2R^{-/-}$ BTME since increased IFN-γ production by CB_2R-lacking CD8 T (C. Y. Yuan et al., 2021) and NK cells (Ferrini et al., 2017) has been reported. Nonetheless, we cannot rule out the involvement of additional mechanisms regulating PD-L1 expression. For example, it has been reported that lack of CB_2R results in increased IL-6 levels in wound tissue (Ruhl et al., 2021), and this cytokine can activate the JAK/STAT pathway, leading to enhanced stability of PD-L1 (L.-C. Chan et al., 2019). Nonetheless, further research is needed to clarify the precise role of CB_2R in regulating PD-L1 expression.

While the immune system is important in the initiation and progression of TNBC, the involvement of other cell populations cannot be excluded. As observed in the CB_2R^{eGFP} model, CB_2R expression is notably high in CAFs. Indeed, it has been reported that CB_2R is induced in interstitial fibroblasts after TGF-β1 stimulation (L. Zhou et al., 2018), a cytokine that is highly abundant in the BTME (W. Chen, Qin, et al., 2018) and is implicated in the differentiation of fibroblasts into CAFs (Shi et al., 2020). Moreover, proteomic analyses from our group suggested a potential interaction between CB_2R and TGF-βR1 (Seijo et al., manuscript submitted), which could facilitate the response to the cytokine and the transition to a pro-fibrotic phenotype. Supporting this notion, Zhou and colleagues reported that knockdown of CB_2R inhibited TGF-β1 fibrogenic responses and that CB_2R-lacking mice exhibited a significant attenuation in the renal expression of the CAF marker α-SMA in a model of renal fibrosis (L. Zhou et al., 2018). However, in a model of liver fibrosis, $CB_2R^{-/-}$ animals showed an upregulation in α-SMA expression (Long et al., 2021). Although

contradictory, these results show that CB_2R plays a context-dependent role in the activation of CAFs. Although it is beyond the scope of this thesis, given the importance of this population within the BTME, it would be of interest to investigate whether CB_2R expression in CAFs is similar to that of fibroblasts in non-transformed breast tissue, and the functional relevance of those differences (if any). Moreover, it would be interesting to characterise the predominant CAF profile in the absence of CB_2R and to determine whether this could help explain the differences in tumour growth observed in our model.

Metastasis remains the leading cause of cancer-related mortality, accounting for approximately 90 % of patient deaths (Bonotto et al., 2014). Despite treatable—primarily with the aim of improving quality-adjusted life expectancy—the metastatic disease is still considered incurable with currently available therapeutic options (Harbeck et al., 2019). Therefore, it is urgent to understand the factors that regulate metastatic progression and that can help clinicians to stratify patients according to their risk of metastatic spread. In our study, we observed that 85.7 % of CB_2R-expressing female mice developed lung metastases, compared to only 50 % of CB_2R-lacking animals, pointing to a pro-metastatic role of stromal CB_2R in our model. However, we found no differences in the immune cell percentages or in the expression of key immunosurveillance markers within established metastatic lesions. This points to a pro-metastatic effect of CB_2R occurring earlier in the metastatic cascade, but future experiments will be necessary to determine whether metastasis is impaired or merely delayed in the absence of CB_2R. Nonetheless, we cannot exclude the contribution of other cell types distinct to those of the immune system to the metastatic process. Notably, endothelial cells have been shown to upregulate CB_2R expression under pathological conditions, such as glioblastoma (Schley et al., 2009) and HIV-infected brains (Ramirez et al., 2012), compared to healthy controls. This is consistent with our observation of high CB_2R expression in endothelial cells of AT3 tumours generated in CB_2R^{eGFP} females. Activation of CB_2R in endothelial cells has been demonstrated to prevent barrier leakiness, increase tight junction integrity and reduce the expression of adhesion molecules such as ICAM-1 (Rajesh et al., 2007; Ramirez et al., 2012). In the absence of CB_2R this may translate into reduced protection to cancer cell extravasation, but, paradoxically, in our model, $CB_2R^{-/-}$ animals exhibited delayed lung metastases. This result suggests that early steps in the metastasis cascade—such as pre-metastatic niche formation or tumour cell survival in circulation—may be more critically affected by CB_2R deletion than the extravasation itself. It is possible that the expression of CB_2R results in the formation of a more propitious pre-metastatic niche that favours initial seeding, a hypothesis supported by our finding of low levels of pro-metastatic soluble factors (as determined by cytokine array) in CB_2R-lacking primary tumours compared to CB_2R-expressing control. Alternatively, diminished immune-mediated elimination of circulating tumour cells may exist in $CB_2R^{+/+}$ animals, a hypothesis supported by the observed trend towards increased killing capacity in CB_2R-lacking splenocytes from non-tumour bearing mice activated with ConA compared to their WT counterparts. However, future experiments assessing earlier points in the metastatic cascade are needed to help clarify the role of CB_2R in the metastatic process.

It is important to note that in our mouse model, genetic deletion of CB_2R occurred in all cells of the organism, except for the tumour cells that we injected. Therefore, the observed changes in immune infiltration and the expression of immunosurveillance markers may not be exclusively attributed to the lack of CB_2R in these populations, but rather to the contribution of other cell types. To specifically analyse the role of CB_2R in antitumour

immunity, we could use mice expressing the Cre-recombinase under the control of an immune cell-specific promoter and cross them with our CB_2R^{eGFP} mouse model. For a broad overview of CB_2R in the immune system, we could employ the CD45-Cre mouse (Yang et al., 2008) as it allows genetic deletion in all hematopoietic cells. However, given the expression pattern of CB_2R and its role in antitumour immunity, we considered particularly interesting to analyse specifically cytotoxic T cells. We currently have access to the CD4-Cre mouse, but in this model the deletion of CB_2R occurs in both CD4 and CD8 T cells, since these cells pass through a double-positive ($CD4^+CD8^+$) stage during thymic development (Y. Xiong & Bosselut, 2012). To achieve a more specific deletion only in cytotoxic T cells, the $E8I^{Cre.GFP}$ mouse could be used (Maekawa et al., 2008). However, our results indicate that the deletion of CB_2R, both globally and specifically in T cells ($CB_2R^{-/-}$ and CD4-$CB_2R^{-/-}$ mouse models, respectively) leads to alterations in immune cell homeostasis, complicating the interpretations of the role of CB_2R within the BTME. In addition, and to avoid developmental alterations, it would be necessary to employ inducible gene deletion models, allowing temporal control of CB_2R deletion. An example of this approach is the tamoxifen-inducible $E8I^{CreERT2.GFP}$ mouse (C. Liu et al., 2020), which would allow us to delete CB_2R prior to tumour cell injection or even during tumour progression.

Collectively, and as summarised in **Figure D1**, our results indicate that stromal CB_2R plays a pro-tumoural role in TNBC by promoting tumour growth, in part through the establishment of a BTIME less permissive to effective antitumour responses—reflected in a reduced infiltration of immune cell associated with better BC patient outcomes and a predominance of cytotoxic cells (mainly CD8 T cells) that appear to be more dysfunctional or exhausted. Furthermore, CB_2R also appears to facilitate lung metastasis in TNBC, probably by promoting the colonisation of distant secondary organs by cancer cells.

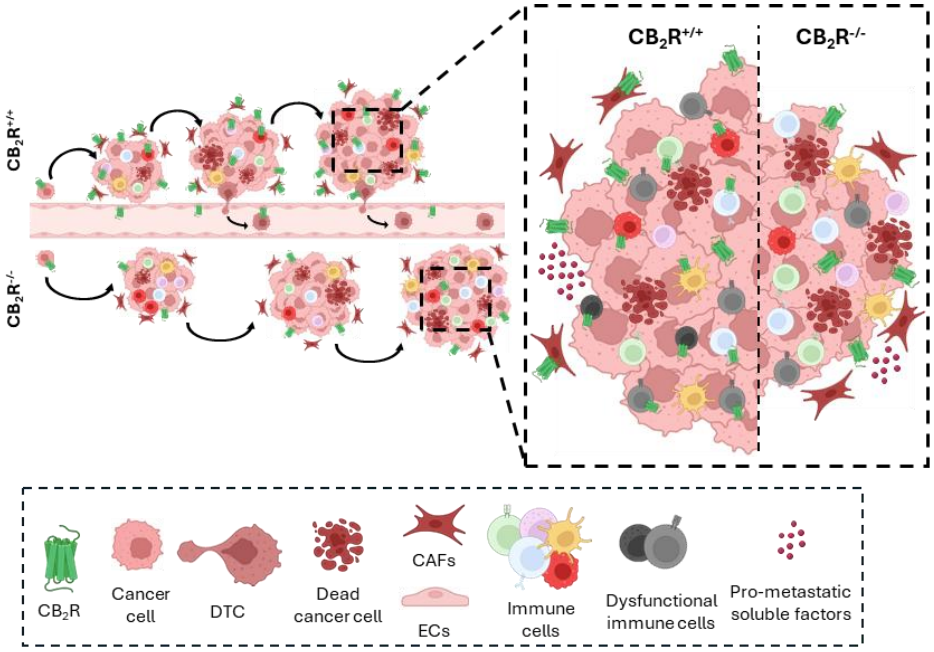

Figure D1. Role of TME-CB_2R in the progression of TNBC. CB_2R expressed by the stroma accelerates TNB tumour growth, probably by limiting the intratumoural infiltration of immune cells generally associated to improved prognosis in BC patients and by promoting the dysfunction of cytotoxic cells. Moreover, CB_2R facilitates lung metastasis without significantly altering the immune landscape of the established metastatic lesions. CAFs: cancer-associated fibroblasts; DTC: disseminated tumour cell; EC: endothelial cell.

ANALYSIS OF THE SIMULTANEOUS TARGETING OF CB_2R IN IMMUNE AND CANCER CELLS

Pharmacological activation of CB_2R expressed by cancer cells has demonstrated to trigger potent antitumoural responses in preclinical models of BC (Blasco-Benito et al., 2018; Caffarel et al., 2006, 2010), glioma (C. Sánchez et al., 1998) and skin cancer (Casanova et al., 2003), among others. In this thesis, we have demonstrated that both the selective CB_2R agonist (HU-308) and the partial CB_1R/CB_2R agonist (THC) decreased the viability of murine TNBC AT3 cells in a concentration-dependent manner. Notably, the concentrations required in our experiments were higher than those reported by other groups to achieve comparable reductions in cell viability. A plausible explanation for these discrepancies lies in differences in experimental conditions, mainly in the presence or absence of serum in the culture medium. It should be considered that serum contains proteins and growth factors that may interact with cannabinoids, potentially modifying their bioavailability or interfering with their mechanism of action, therefore complicating the interpretation of the results. In our study, cells were maintained in medium supplemented with 10 % FBS throughout the treatment period, whereas in other studies, serum was reduced to minimal levels (Caffarel et al., 2006, 2010; Casanova et al., 2003) or even eliminated (Blasco-Benito et al., 2018; C. Sánchez et al., 1998). In fact, as early as 1992, Zheng and colleagues reported that THC ability to inhibit TNFα production by macrophages depended on the amount of serum proteins in the medium (Zheng et al., 1992). Subsequently, other groups confirmed that this rationale was also applicable to the antitumoural action of cannabinoids indicating that, in the presence of serum, higher cannabinoid concentrations were needed to achieve the same effect (Jacobsson et al., 2000; McKallip, Lombard, Fisher, et al., 2002). Nonetheless, and although it has been reported that the viability of non-tumoural cells remains mainly unaffected by cannabinoid challenge (Velasco et al., 2016), it would be necessary to confirm that the concentrations of HU-308 and THC used in this thesis do not compromise the viability of non-tumoural cells.

Cannabinoids have also been reported to exert immunosuppressive effects when targeting CB_2R expressed by immune cells. For example, THC has been shown to induce apoptosis in splenocytes via CB_2R (McKallip et al., 2002) or significantly inhibit proliferation, effector function, and JAK/STAT signalling in WT T cells, but no in CB_2R-lacking T cells (X. Xiong et al., 2022). Several CB_2R-selective agonists also display immunosuppressive properties: JWH-133 reduced proliferation and pro-inflammatory cytokine production in $CB_2R^{+/+}$, but not in $CB_2R^{-/-}$ T cells (Maresz et al., 2007); JWH-015 inhibited the proliferative response to ConA stimulation via CB_2R (Lombard et al., 2007); and HU-308 decreased NO and TNF release in activated microglia in a CB_2R-dependent mechanism (Young et al., 2024). In our study, both HU-308 and THC exert immunosuppressive-like effects in experimental models simulating different stages of immunosurveillance, primarily by reducing cell viability and interfering with JAK/STAT signalling. Intriguingly, these effects were produced in cells expressing and lacking CB_2R, clearly pointing to a CB_2R-independent action. These findings are difficult to explain, and discrepancies regarding CB_2R involvement may be attributed to the high concentrations used in our models, which could result in cannabinoid action through a yet unidentified off-target. Indeed, immunomodulatory effects of THC independent of CB_2R have been described. For instance, treatment of both WT and $CB_1R/CB_2R^{-/-}$ T cells with THC during the elicitation phase (for the generation of effector cytotoxic T cells) decreased viability, induced CD69 expression and reduced CD25 (a late activation marker) in both

genotypes, possibly mediated by calcium signalling perturbation via TRPC1 activation (Karmaus et al., 2012). These results support our own findings of reduced viability and PD-1 in splenocytes treated with ConA and THC, although we did not observe significant changes in CD69 expression when added together. Further evidence of CB_2R-independent immunosuppression by THC has been provided by Springs and colleagues, who showed that treatment of WT and $CB_1R/CB_2R^{-/-}$ splenocytes with THC prior to activation resulted in a concentration-dependent decrease in proliferation and IFN-γ production, irrespective of genotype (Springs et al., 2008). Notably, and although the implication of CB_2R was not assessed, the ability of THC to inhibit IFN-γ release appears to depend on the timing of exposure: THC reduces IFN-γ production when added during cell activation, but not after (Henriquez et al., 2020). This is consistent with our results, where cytokine production is affected when cannabinoids are added during ConA stimulation, but not when added post-activation, and with the observations made by Springs and colleagues, who reported that only naive CD8 T cells, but not differentiated cytotoxic T cells, were sensitive to THC-mediated suppression of cytotoxic function (Springs et al., 2008).

In contrast, CB_2R-independent immunosuppressive effects have not been reported for HU-308. In fact, it is considered one of the most selective CB_2R agonists currently available. However, a comprehensive characterisation of various cannabinoid ligands by Soethoudt and colleagues revealed that HU-308 may interact with some off-targets, primarily at the micromolar range (Soethoudt et al., 2017). For example, HU-308 was found to bind to cholecystokinin receptors, whose activation inhibits mitogen-induced proliferation and reduces pro-inflammatory cytokine expression in THP-1 macrophage-like cells *in vitro* (Reich & Hölscher, 2024). HU-308 may also interact with the dopamine transporter, the blockade of which enhances LPS-induced production of pro-inflammatory cytokines (Gopinath et al., 2023). Additionally, HU-308 can act on calcium channels such as TRPV1, whose activation promotes calcium influx, which can ultimately lead to apoptosis (J. Chen et al., 2024). Given these potential off-targets, it would be interesting to assess their expression in $CB_2R^{-/-}$ vs $CB_2R^{+/+}$ immune cells, and to determine whether any of them contributes to the observed effects in our experimental models.

It is important to emphasise that in our experimental models, cannabinoids were used at relatively high concentrations, so we cannot rule out the possibility of the observed effects resulting from their interaction with cellular membranes and not mediated by their interaction with potential off-target receptors. As lipid-soluble compounds, cannabinoids may alter the structure, biophysical and physicochemical properties of the plasma membrane (Oz et al., 2022). For example, they may disrupt membrane microdomains (lipid rafts), leading to alterations in the localisation and function of critical signalling proteins. Moreover, they can alter membrane permeability, disrupting ionic gradients—essential for cell signalling—and leading to cell death if not adequately regulated (Itri et al., 2014).

Most of the *in vivo* research showing antitumour actions of cannabinoids has been performed in immunodeficient mice. In immunocompetent animal models, however, cannabinoids have been shown to exert both pro- and antitumoural effects. Specifically in BC, our group previously reported a reduction in tumour growth in FVB/NJ MMTVneu female mice treated with THC and JWH-133, without observing significant differences in immune infiltration (Caffarel et al., 2010). On the contrary, McKallip and collaborators reported a pro-tumoural and pro-metastatic effect of THC associated with an impairment of antitumour immunity (McKallip et al., 2005). In our cancer-splenocyte co-culture

experimental model, we observed that the antitumoural action of cannabinoids prevails over their immunosuppressive effects. Notably, this effect appears to be independent of CB_2R expression by immune cells. Although our flow cytometry analyses are still preliminary and should be complemented by experiments using CB_2R-lacking cells, they indicate that cannabinoids may decrease immune cell viability and upregulate PD-1 expression, potentially promoting a more immunosuppressive microenvironment. However, it should be noted that PD-1 expression in total immune cells does not always accurately reflect its expression in T cells. Indeed, it has been reported a diminished expression of PD-1 in specific subsets of CD8 T cells in cannabis users (Falcinelli et al., 2022). Additionally, our results indicate that cannabinoids may facilitate immune evasion by upregulating PD-L1 in cancer cells, while not affecting their visibility by the immune system, as reflected by the absence of changes in MHC-I levels. However, it has been reported that cannabinoids may induce MHC-I expression in metastatic BC cells *in vitro* (Dada et al., 2023)—a result we have been able to replicate in AT3 cells when cultured alone, but not in co-culture conditions. Taking all these results into account, it would be of great interest to confirm them in an *in vivo* model, analysing whether cannabinoids may influence AT3-derived tumour progression in $CB_2R^{+/+}$ and $CB_2R^{-/-}$ mice. In such studies we would also assess potential changes in the immune composition of the BTME—both in terms of cell percentage and phenotype, as well as expression of immunosurveillance markers—and determine if cannabinoids can prevent the downregulation of MHC-I molecules by cancer cells during tumour progression—an event that has been associated with worse outcomes in BC (Taylor & Balko, 2022).

Although our results are promising and point to the safety of cannabinoids as antitumour tools, we are aware of the inherent limitations of our co-culture model. By pre-treating immune cells with ConA, we presumably achieved polyclonal T cell activation that is not specific of tumour antigens. This translates into an indiscriminate tumour cell killing that could be mediated by different mechanisms such as degranulation of cytotoxic granules, release of cytokines with cytotoxic or pro-apoptotic effects, or interaction between T-cell expressed FasL and tumoural Fas receptor. To more accurately recapitulate the specific antitumour immune response that may occur *in vivo*, we could isolate splenocytes and co-culture them in a first step with irradiated, non-proliferative cancer cells, generating antigen-specific effector T cells that would be then co-cultured with proliferating tumour cells (Karmaus et al., 2012). Alternately, we could employ MHC-restricted systems, such as the OVA/OT-I system, which is based in the specific recognition of target cells engineered to overexpress the ovalbumin (OVA) peptide by CD8 T cells isolated from OT-I mice and expressing a transgenic TCR specific for the OVA peptide (Clarke et al., 2000). For human cells, an HLA-restricted system (analogous to the MHC-restricted system used in mouse immunology) could be used. For example, target cells could be engineered to overexpress the NY-ESO-1 peptide, while T cells are transduced with a transgenic TCR direct against it (Thomas et al., 2018). However, all these *in vitro* models lack other key components of the TME, such as fibroblasts or endothelial cells. To better reproduce this complex ecosystem, we could alternatively employ the Alginate-based tissue engineering (ALTEN) technology, that allows long-term culture of tissue explants while preserving their original characteristics (Law et al., 2022).

Cannabinoids, either as pure compounds or as botanical cannabis extracts, have been demonstrated in preclinical models not to interfere with standard BC therapies (Blasco-Benito et al., 2018) or even to synergise with chemotherapeutic agents or radiotherapy in

brain cancer models (Scott et al., 2014, 2017; Torres et al., 2011). Indeed, this synergy has been suggested in a clinical study performed in patients with recurrent glioblastoma treated with temozolomide in combination with nabiximols (Twelves et al., 2021), although definitive confirmation may be obtained in the ARISTOCRAT clinical trial (ClinicalTrials.Gov: NCT05629702—currently recruiting) (Bhaskaran et al., 2024). However, the possible interaction between cannabinoids and immunotherapy remains a subject of debate, mainly due to the immunosuppressive actions of cannabinoids. In this line, Xiong and colleagues reported that THC reduced the therapeutic effect of PD-1 blockade in preclinical models of cancer (X. Xiong et al., 2022), and two observational studies by Taha and Bar-Sela groups indicated that the use of cannabis during immunotherapy was associated with worsen outcomes (Bar-Sela et al., 2020; Taha et al., 2019). On the contrary, preclinical and clinical data obtained by Waissengrin and collaborators suggest no deleterious effect of cannabis on the activity of anti-PD-1 immunotherapy (Waissengrin et al., 2023). Moreover, a recent study by Piper and colleagues indicated that the results obtained by Taha and Bar-Sela should be interpreted with caution due to substantial inaccuracies that could affect statistical conclusions (Piper et al., 2024).

In this context, our results suggest that cannabinoids impact on the antitumour activity of immune cells is concentration dependent. Indeed, at low concentrations we did not observe a clear interference with the cytotoxic capacity of immune cells, whereas at higher concentrations, the antitumour effect seems to predominate over immunomodulation. This observation is particularly interesting considering the data published by Taha and collaborators, as they reported that patients consuming cannabis products containing high THC content exhibited better response rates to immunotherapy than those consuming low-THC products (Taha et al., 2019). Thus, it is reasonable to consider whether, under certain conditions, the combination of cannabinoids and immunotherapy could be beneficial. To further evaluate this possibility, we could employ *in vivo* models in which different concentrations of cannabinoids are combined with immunotherapy, using various treatment schedules. For example, given that AT3 cells have been reported to be resistant to anti-PD-1 therapy, it would be interesting to investigate whether concomitant treatment with cannabinoids and immunotherapy may improve treatment response compared to either monotherapy. Alternatively, a sequential treatment approach—initiating therapy with cannabinoids prior to immunotherapy—could be tested to evaluate if such regimen sensitises to immune checkpoint blockade. Moreover, the use of $CB_2R^{+/+}$ and $CB_2R^{-/-}$ mice in these experiments could help elucidate whether the response to combined therapy is influenced by CB_2R expression in the BTME and clarify whether stromal CB_2R could serve as a predictive biomarker for therapeutic response. These strategies may help clarify the possible interactions between cannabinoids and immunotherapy and may help identify optimal conditions for therapeutic synergy, leading to the development of more effective combined cancer treatments.

Collectively, and as summarised in **Figure D2**, our results indicate that cannabinoids exert immunosuppressive-like effects via yet unidentified targets distinct from CB_2R. However, their antitumoural action on cancer cells prevails over its immunomodulatory role when tumour and immune cells coexist.

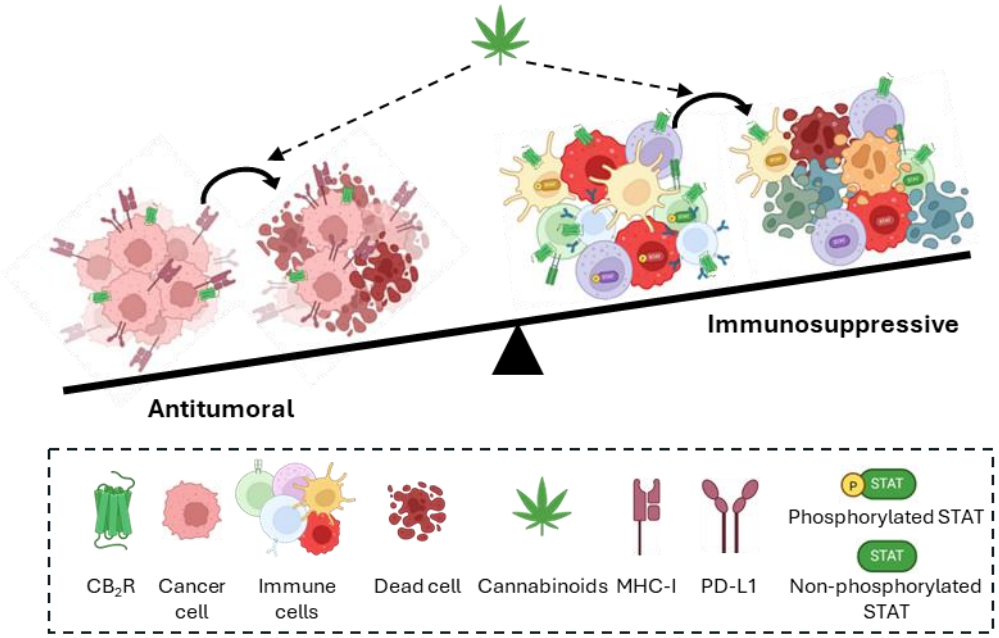

Figure D2. Effect of cannabinoids on tumour and immune cells. Cannabinoids, through the activation of CB_2R, induce cancer cell death. In immune cells, however, they exert immunosuppressive-like effects, primarily through the reduction of cell viability and interference with the JAK/STAT signalling pathway, in a CB_2R-independent mechanism. Notably, within the context of tumour-immune cell interactions (as would occur in a breast tumour), the antitumoural effects of cannabinoids outweigh their immunosuppressive actions.

CONCLUSIONS

CONCLUSIONS

The results obtained in this thesis allow us to draw the following conclusions:

- CB_2R is expressed by immune cells, including those in the BTME, where it modulates antitumour immunity by regulating the levels of immunosurveillance markers. Regarding the PD-1/PD-L1 axis, CB_2R may increase the threshold required for CD8 T cell activation and simultaneously contribute to the establishment of a more immunosuppressive microenvironment through the induction of PD-L1 expression in different immune cell populations.

- Stromal CB_2R has a pro-tumoural role in TNBC, contributing to the establishment of a BTIME that is less conducive to effective antitumoural immune responses in the primary tumour—mainly by limiting the infiltration of immune cells associated with improved outcomes in BC patients and promoting the dysfunction of cytotoxic cells (both NK and CD8 T cells). Moreover, TME-CB_2R has a pro-metastatic role in TNBC facilitating the metastatic colonisation of secondary organs by tumour cells.

- Cannabinoids exert immunosuppressive-like effects via CB_2R-independent mechanisms and regardless the activation state of immune cells. However, in contexts where cancer-immune cell interactions occur (as in actual tumours), the antitumour effect predominates over immunomodulation. This reinforces the potential of cannabinoids as safe and effective antitumour agents.

REFERENCES

Abdou, Y., Goudarzi, A., Yu, J. X., Upadhaya, S., Vincent, B., & Carey, L. A. (2022). Immunotherapy in triple negative breast cancer: beyond checkpoint inhibitors. *Npj Breast Cancer*, *8*(1), 121.

Acharya, N., Penukonda, S., Shcheglova, T., Hagymasi, A. T., Basu, S., & Srivastava, P. K. (2017). Endocannabinoid system acts as a regulator of immune homeostasis in the gut. *Proceedings of the National Academy of Sciences*, *114*(19), 5005–5010.

Adams, R. (1942). Marihuana: Harvey Lecture, February 19, 1942. *Bulletin of the New York Academy of Medicine*, *18*(11), 705–730.

Adams, S., Goldstein, L. J., Sparano, J. A., Demaria, S., & Badve, S. S. (2015). Tumor infiltrating lymphocytes (TILs) improve prognosis in patients with triple negative breast cancer (TNBC). *OncoImmunology*, *4*(9), e985930.

Adams, S., Gray, R. J., Demaria, S., Goldstein, L., Perez, E. A., Shulman, L. N., Martino, S., Wang, M., Jones, V. E., Saphner, T. J., Wolff, A. C., Wood, W. C., Davidson, N. E., Sledge, G. W., Sparano, J. A., & Badve, S. S. (2014). Prognostic Value of Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancers From Two Phase III Randomized Adjuvant Breast Cancer Trials: ECOG 2197 and ECOG 1199. *Journal of Clinical Oncology*, *32*(27), 2959–2966.

Ali, H. R., Provenzano, E., Dawson, S.-J., Blows, F. M., Liu, B., Shah, M., Earl, H. M., Poole, C. J., Hiller, L., Dunn, J. A., Bowden, S. J., Twelves, C., Bartlett, J. M. S., Mahmoud, S. M. A., Rakha, E., Ellis, I. O., Liu, S., Gao, D., Nielsen, T. O., … Caldas, C. (2014). Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients. *Annals of Oncology*, *25*(8), 1536–1543.

Alsaab, H. O., Sau, S., Alzhrani, R., Tatiparti, K., Bhise, K., Kashaw, S. K., & Iyer, A. K. (2017). PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Frontiers in Pharmacology*, *8*.

Amens, J. N., Bahçecioglu, G., & Zorlutuna, P. (2021). Immune System Effects on Breast Cancer. *Cellular and Molecular Bioengineering*, *14*(4), 279–292.

Andradas, C., Blasco-Benito, S., Castillo-Lluva, S., Dillenburg-Pilla, P., Diez-Alarcia, R., Juanes-García, A., García-Taboada, E., Hernando-Llorente, R., Soriano, J., Hamann, S., Wenners, A., Alkatout, I., Klapper, W., Rocken, C., Bauer, M., Arnold, N., Quintanilla, M., Megías, D., Vicente-Manzanares, M., … Sánchez, C. (2016). Activation of the orphan receptor GPR55 by lysophosphatidylinositol promotes metastasis in triple-negative breast cancer. *Oncotarget*, *7*(30), 47565–47575.

Andradas, C., Caffarel, M. M., Pérez-Gómez, E., Salazar, M., Lorente, M., Velasco, G., Guzmán, M., & Sánchez, C. (2011). The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. *Oncogene*, *30*(2), 245–252.

Arianfar, E., Shahgordi, S., & Memarian, A. (2021). Natural Killer Cell Defects in Breast Cancer: A Key Pathway for Tumor Evasion. *International Reviews of Immunology*, *40*(3), 197–216.

Arnold, M., Morgan, E., Rumgay, H., Mafra, A., Singh, D., Laversanne, M., Vignat, J., Gralow, J. R., Cardoso, F., Siesling, S., & Soerjomataram, I. (2022). Current and future burden of breast cancer: Global statistics for 2020 and 2040. *The Breast*, *66*, 15–23.

Arole, V., Nitta, H., Wei, L., Shen, T., Parwani, A. V., & Li, Z. (2021). M2 tumor-associated macrophages play important role in predicting response to neoadjuvant chemotherapy in triple-negative breast carcinoma. *Breast Cancer Research and Treatment*, *188*(1), 37–42.

Atzeni, F., Schena, M., Ongari, A. M., Carrabba, M., Bonara, P., Minonzio, F., & Capsoni, F. (2002). Induction of CD69 activation molecule on human neutrophils by GM-CSF, IFN-γ, and IFN-α. *Cellular Immunology*, *220*(1), 20–29.

Badr, N. M., McMurray, J. L., Danial, I., Hayward, S., Asaad, N. Y., Abd El-Wahed, M. M., Abdou, A. G., Serag El-Dien, M. M., Sharma, N., Horimoto, Y., Sircar, T., Vidya, R., Hoar, F., Rea, D., Jones, J. L., Stevens, A., Spooner, D., Merard, R., Lewis, P., … Shaaban, A. M. (2023). Characterization of the Immune Microenvironment in Inflammatory Breast Cancer Using Multiplex Immunofluorescence. *Pathobiology*, *90*(1), 31–43.

Barker, H. A., Bhimani, S., Tirado, D., Lemos, L. N., Roesch, L. F. W., & Ferraro, M. J. (2025). *Cannabinoid receptor deficiencies drive immune response dynamics in Salmonella infection*.

Bar-Sela, G., Cohen, I., Campisi-Pinto, S., Lewitus, G. M., Oz-Ari, L., Jehassi, A., Peer, A., Turgeman, I., Vernicova, O., Berman, P., Wollner, M., Moskovitz, M., & Meiri, D. (2020). Cannabis Consumption Used by Cancer Patients during Immunotherapy Correlates with Poor Clinical Outcome. *Cancers*, *12*(9), 2447.

Barsoum, I. B., Hamilton, T. K., Li, X., Cotechini, T., Miles, E. A., Siemens, D. R., & Graham, C. H. (2011). Hypoxia Induces Escape from Innate Immunity in Cancer Cells via Increased Expression of ADAM10: Role of Nitric Oxide. *Cancer Research*, *71*(24), 7433–7441.

Bartoschek, M., Oskolkov, N., Bocci, M., Lövrot, J., Larsson, C., Sommarin, M., Madsen, C. D., Lindgren, D., Pekar, G., Karlsson, G., Ringnér, M., Bergh, J., Björklund, Å., & Pietras, K. (2018). Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing. *Nature Communications*, *9*(1), 5150.

Basu, S., Ray, A., & Dittel, B. N. (2011). Cannabinoid Receptor 2 Is Critical for the Homing and Retention of Marginal Zone B Lineage Cells and for Efficient T-Independent Immune Responses. *The Journal of Immunology*, *187*(11), 5720–5732.

Basu, S., Ray, A., & Dittel, B. N. (2013). Cannabinoid Receptor 2 (CB2) Plays a Role in the Generation of Germinal Center and Memory B Cells, but Not in the Production of Antigen-Specific IgG and IgM, in Response to T-dependent Antigens. *PLoS ONE*, *8*(6), e67587.

Bates, G. J., Fox, S. B., Han, C., Leek, R. D., Garcia, J. F., Harris, A. L., & Banham, A. H. (2006). Quantification of Regulatory T Cells Enables the Identification of High-Risk Breast Cancer Patients and Those at Risk of Late Relapse. *Journal of Clinical Oncology*, *24*(34), 5373–5380.

Beltra, J.-C., Manne, S., Abdel-Hakeem, M. S., Kurachi, M., Giles, J. R., Chen, Z., Casella, V., Ngiow, S. F., Khan, O., Huang, Y. J., Yan, P., Nzingha, K., Xu, W., Amaravadi, R. K.,

Xu, X., Karakousis, G. C., Mitchell, T. C., Schuchter, L. M., Huang, A. C., & Wherry, E. J. (2020). Developmental Relationships of Four Exhausted CD8+ T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. *Immunity*, *52*(5), 825-841.e8.

Bense, R. D., Sotiriou, C., Piccart-Gebhart, M. J., Haanen, J. B. A. G., van Vugt, M. A. T. M., de Vries, E. G. E., Schröder, C. P., & Fehrmann, R. S. N. (2017). Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for Disease Outcome in Breast Cancer. *Journal of the National Cancer Institute*, *109*(1), djw192.

Beziaud, L., Young, C. M., Alonso, A. M., Norkin, M., Minafra, A. R., & Huelsken, J. (2023). IFNγ-induced stem-like state of cancer cells as a driver of metastatic progression following immunotherapy. *Cell Stem Cell*, *30*(6), 818-831.e6.

Bhaskaran, D., Savage, J., Patel, A., Collinson, F., Mant, R., Boele, F., Brazil, L., Meade, S., Buckle, P., Lax, S., Billingham, L., & Short, S. C. (2024). A randomised phase II trial of temozolomide with or without cannabinoids in patients with recurrent glioblastoma (ARISTOCRAT): protocol for a multi-centre, double-blind, placebo-controlled trial. *BMC Cancer*, *24*(1), 83.

Blasco-Benito, S., Moreno, E., Seijo-Vila, M., Tundidor, I., Andradas, C., Caffarel, M. M., Caro-Villalobos, M., Urigüen, L., Diez-Alarcia, R., Moreno-Bueno, G., Hernández, L., Manso, L., Homar-Ruano, P., McCormick, P. J., Bibic, L., Bernadó-Morales, C., Arribas, J., Canals, M., Casadó, V., … Sánchez, C. (2019). Therapeutic targeting of HER2–CB 2 R heteromers in HER2-positive breast cancer. *Proceedings of the National Academy of Sciences*, *116*(9), 3863–3872.

Blasco-Benito, S., Seijo-Vila, M., Caro-Villalobos, M., Tundidor, I., Andradas, C., García-Taboada, E., Wade, J., Smith, S., Guzmán, M., Pérez-Gómez, E., Gordon, M., & Sánchez, C. (2018). Appraising the “entourage effect”: Antitumor action of a pure cannabinoid versus a botanical drug preparation in preclinical models of breast cancer. *Biochemical Pharmacology*, *157*, 285–293.

Blázquez, C., Carracedo, A., Barrado, L., José Real, P., Luis Fernández-Luna, J., Velasco, G., Malumbres, M., Guzmán, M., Blázquez, C., Carracedo, A., Barrado, L., José Real, P., Luis Fernández-Luna, J., Velasco, G., Malumbres, M., & Guzmán, M. (2006). Cannabinoid receptors as novel targets for the treatment of melanoma. *The FASEB Journal*, *20*(14), 2633–2635.

Blázquez, C., Casanova, M. L., Planas, A., Gómez del Pulgar, T., Villanueva, C., Fernández-Aceñero, M. J., Aragonés, J., Huffman, J. W., Jorcano, J. L., & Guzmán, M. (2003). Inhibition of tumor angiogenesis by cannabinoids. *The FASEB Journal*, *17*(3), 1–16.

Blázquez, C., González-Feria, L., Álvarez, L., Haro, A., Casanova, M. L., & Guzmán, M. (2004). Cannabinoids Inhibit the Vascular Endothelial Growth Factor Pathway in Gliomas. *Cancer Research*, *64*(16), 5617–5623.

Blázquez, C., Salazar, M., Carracedo, A., Lorente, M., Egia, A., González-Feria, L., Haro, A., Velasco, G., & Guzmán, M. (2008). Cannabinoids Inhibit Glioma Cell Invasion by Down-regulating Matrix Metalloproteinase-2 Expression. *Cancer Research*, *68*(6), 1945–1952.

Bloom, H. J. G., & Richardson, W. W. (1957). Histological Grading and Prognosis in Breast Cancer. *British Journal of Cancer*, *11*(3), 359–377.

Bochet, L., Lehuédé, C., Dauvillier, S., Wang, Y. Y., Dirat, B., Laurent, V., Dray, C., Guiet, R., Maridonneau-Parini, I., Le Gonidec, S., Couderc, B., Escourrou, G., Valet, P., & Muller, C. (2013). Adipocyte-Derived Fibroblasts Promote Tumor Progression and Contribute to the Desmoplastic Reaction in Breast Cancer. *Cancer Research*, *73*(18), 5657–5668.

Boieri, M., Malishkevich, A., Guennoun, R., Marchese, E., Kroon, S., Trerice, K. E., Awad, M., Park, J. H., Iyer, S., Kreuzer, J., Haas, W., Rivera, M. N., & Demehri, S. (2022). CD4+ T helper 2 cells suppress breast cancer by inducing terminal differentiation. *Journal of Experimental Medicine*, *219*(7).

Bonotto, M., Gerratana, L., Poletto, E., Driol, P., Giangreco, M., Russo, S., Minisini, A. M., Andreetta, C., Mansutti, M., Pisa, F. E., Fasola, G., & Puglisi, F. (2014). Measures of Outcome in Metastatic Breast Cancer: Insights From a Real-World Scenario. *The Oncologist*, *19*(6), 608–615.

Braumüller, H., Wieder, T., Brenner, E., Aßmann, S., Hahn, M., Alkhaled, M., Schilbach, K., Essmann, F., Kneilling, M., Griessinger, C., Ranta, F., Ullrich, S., Mocikat, R., Braungart, K., Mehra, T., Fehrenbacher, B., Berdel, J., Niessner, H., Meier, F., … Röcken, M. (2013). T-helper-1-cell cytokines drive cancer into senescence. *Nature*, *494*(7437), 361–365.

Braun, M., Khan, Z. T., Khan, M. B., Kumar, M., Ward, A., Achyut, B. R., Arbab, A. S., Hess, D. C., Hoda, Md. N., Baban, B., Dhandapani, K. M., & Vaibhav, K. (2018). Selective activation of cannabinoid receptor-2 reduces neuroinflammation after traumatic brain injury via alternative macrophage polarization. *Brain, Behavior, and Immunity*, *68*, 224–237.

Brown, J. R., Wimberly, H., Lannin, D. R., Nixon, C., Rimm, D. L., & Bossuyt, V. (2014). Multiplexed Quantitative Analysis of CD3, CD8, and CD20 Predicts Response to Neoadjuvant Chemotherapy in Breast Cancer. *Clinical Cancer Research*, *20*(23), 5995–6005.

Buckley, N. E., McCoy, K. L., Mezey, É., Bonner, T., Zimmer, A., Felder, C. C., Glass, M., & Zimmer, A. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB2 receptor. *European Journal of Pharmacology*, *396*(2–3), 141–149.

Buijs, S. M., Braal, C. L., Buck, S. A. J., van Maanen, N. F., van der Meijden-Erkelens, L. M., Kuijper-Tissot van Patot, H. A., Hoop, E. O., Saes, L., van den Boogerd, S. J., Struik, L. E. M., van Rossum-Schornagel, Q. C., Mathijssen, R. H. J., Koolen, S. L. W., & Jager, A. (2023). CBD-oil as a potential solution in case of severe tamoxifen-related side effects. *Npj Breast Cancer*, *9*(1), 63.

Burstein, M. D., Tsimelzon, A., Poage, G. M., Covington, K. R., Contreras, A., Fuqua, S. A. W., Savage, M. I., Osborne, C. K., Hilsenbeck, S. G., Chang, J. C., Mills, G. B., Lau, C. C., & Brown, P. H. (2015). Comprehensive Genomic Analysis Identifies Novel Subtypes and Targets of Triple-Negative Breast Cancer. *Clinical Cancer Research*, *21*(7), 1688–1698.

Cabral, G. A., Toney, D. M., Fischer-Stenger, K., Harrison, M. P., & Marciano-Cabral, F. (1995). Anandamide inhibits macrophage-mediated killing of tumor necrosis factor-sensitive cells. *Life Sciences*, *56*(23–24), 2065–2072.

Caffarel, M. M., Andradas, C., Mira, E., Pérez-Gómez, E., Cerutti, C., Moreno-Bueno, G., Flores, J. M., García-Real, I., Palacios, J., Mañes, S., Guzmán, M., & Sánchez, C. (2010). Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition. *Molecular Cancer*, *9*(1), 196.

Caffarel, M. M., Sarrió, D., Palacios, J., Guzmán, M., & Sánchez, C. (2006). Δ9-Tetrahydrocannabinol Inhibits Cell Cycle Progression in Human Breast Cancer Cells through Cdc2 Regulation. *Cancer Research*, *66*(13), 6615–6621.

Carayon, P., Marchand, J., Dussossoy, D., Derocq, J.-M., Jbilo, O., Bord, A., Bouaboula, M., Galiègue, S., Mondière, P., Pénarier, G., Fur, G. Le, Defrance, T., & Casellas, P. (1998). Modulation and Functional Involvement of CB2 Peripheral Cannabinoid Receptors During B-Cell Differentiation. *Blood*, *92*(10), 3605–3615.

Carlisle, S. J., Marciano-Cabral, F., Staab, A., Ludwick, C., & Cabral, G. A. (2002). Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *International Immunopharmacology*, *2*(1), 69–82.

Carracedo, A., Gironella, M., Lorente, M., Garcia, S., Guzmán, M., Velasco, G., & Iovanna, J. L. (2006). Cannabinoids Induce Apoptosis of Pancreatic Tumor Cells via Endoplasmic Reticulum Stress–Related Genes. *Cancer Research*, *66*(13), 6748–6755.

Carrega, P., Bonaccorsi, I., Di Carlo, E., Morandi, B., Paul, P., Rizzello, V., Cipollone, G., Navarra, G., Mingari, M. C., Moretta, L., & Ferlazzo, G. (2014). CD56brightPerforinlow Noncytotoxic Human NK Cells Are Abundant in Both Healthy and Neoplastic Solid Tissues and Recirculate to Secondary Lymphoid Organs via Afferent Lymph. *The Journal of Immunology*, *192*(8), 3805–3815.

Casanova, M. L., Blázquez, C., Martínez-Palacio, J., Villanueva, C., Fernández-Aceñero, M. J., Huffman, J. W., Jorcano, J. L., & Guzmán, M. (2003). Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. *Journal of Clinical Investigation*, *111*(1), 43–50.

Casbon, A.-J., Reynaud, D., Park, C., Khuc, E., Gan, D. D., Schepers, K., Passegué, E., & Werb, Z. (2015). Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. *Proceedings of the National Academy of Sciences*, *112*(6).

Castaneda, J. T., Harui, A., Kiertscher, S. M., Roth, J. D., & Roth, M. D. (2013). Differential Expression of Intracellular and Extracellular CB2 Cannabinoid Receptor Protein by Human Peripheral Blood Leukocytes. *Journal of Neuroimmune Pharmacology*, *8*(1), 323–332.

Castaneda, J. T., Harui, A., & Roth, M. D. (2017). Regulation of Cell Surface CB2 Receptor during Human B Cell Activation and Differentiation. *Journal of Neuroimmune Pharmacology*, *12*(3), 544–554.

Cazet, A. S., Hui, M. N., Elsworth, B. L., Wu, S. Z., Roden, D., Chan, C.-L., Skhinas, J. N., Collot, R., Yang, J., Harvey, K., Johan, M. Z., Cooper, C., Nair, R., Herrmann, D., McFarland, A., Deng, N., Ruiz-Borrego, M., Rojo, F., Trigo, J. M., … Swarbrick, A. (2018). Targeting stromal remodeling and cancer stem cell plasticity overcomes chemoresistance in triple negative breast cancer. *Nature Communications*, *9*(1), 2897.

Cha, Y. J., & Koo, J. S. (2020). Role of Tumor-Associated Myeloid Cells in Breast Cancer. *Cells*, *9*(8), 1785.

Chan, L.-C., Li, C.-W., Xia, W., Hsu, J.-M., Lee, H.-H., Cha, J.-H., Wang, H.-L., Yang, W.-H., Yen, E.-Y., Chang, W.-C., Zha, Z., Lim, S.-O., Lai, Y.-J., Liu, C., Liu, J., Dong, Q., Yang, Y., Sun, L., Wei, Y., … Hung, M.-C. (2019). IL-6/JAK1 pathway drives PD-L1 Y112 phosphorylation to promote cancer immune evasion. *Journal of Clinical Investigation*, *129*(8), 3324–3338.

Chan, R., Aphivatanasiri, C., Poon, I. K., Tsang, J. Y., Ni, Y., Lacambra, M., Li, J., Lee, C., & Tse, G. M. (2024). Spatial Distribution and Densities of CD103+ and FoxP3+ Tumor Infiltrating Lymphocytes by Digital Analysis for Outcome Prediction in Breast Cancer. *The Oncologist*, *29*(3), e299–e308.

Chen, J., Sun, W., Zhu, Y., Zhao, F., Deng, S., Tian, M., Wang, Y., & Gong, Y. (2024). TRPV1: The key bridge in neuroimmune interactions. *Journal of Intensive Medicine*, *4*(4), 442–452.

Chen, R.-Y., Zhu, Y., Shen, Y.-Y., Xu, Q.-Y., Tang, H.-Y., Cui, N.-X., Jiang, L., Dai, X.-M., Chen, W.-Q., Lin, Q., & Li, X.-Z. (2023). The role of PD-1 signaling in health and immune-related diseases. *Frontiers in Immunology*, *14*.

Chen, W., Hoffmann, A. D., Liu, H., & Liu, X. (2018). Organotropism: new insights into molecular mechanisms of breast cancer metastasis. *Npj Precision Oncology*, *2*(1), 4.

Chen, W., Qin, Y., & Liu, S. (2018). Cytokines, breast cancer stem cells (BCSCs) and chemoresistance. *Clinical and Translational Medicine*, *7*(1).

Chen, X., Wang, Y., Nelson, D., Tian, S., Mulvey, E., Patel, B., Conti, I., Jaen, J., & Rollins, B. J. (2016). CCL2/CCR2 Regulates the Tumor Microenvironment in HER-2/neu-Driven Mammary Carcinomas in Mice. *PLOS ONE*, *11*(11), e0165595.

Cheng, X. (2024). A Comprehensive Review of HER2 in Cancer Biology and Therapeutics. *Genes*, *15*(7), 903.

Cheng, Y. S. R., Ridnour, L., Somasundaram, V., Bhattacharyya, D., McGinity, C., McVicar, D., Anderson, S., Lockett, S., & Wink, D. (2022). Abstract 3804: Interferon gamma induces NOS2 and COX2 expression in estrogen receptor negative breast cancer leading to immunosuppressive tumor microenvironment. *Cancer Research*, *82*(12_Supplement), 3804–3804.

Chiurchiù, V., Battistini, L., & Maccarrone, M. (2015). Endocannabinoid signalling in innate and adaptive immunity. *Immunology*, *144*(3), 352–364.

Chiurchiù, V., Cencioni, M. T., Bisicchia, E., De Bardi, M., Gasperini, C., Borsellino, G., Centonze, D., Battistini, L., & Maccarrone, M. (2013). Distinct modulation of human

myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis. *Annals of Neurology*, *73*(5), 626–636.

Cipriano, M., Häggström, J., Hammarsten, P., & Fowler, C. J. (2013). Association between Cannabinoid CB1 Receptor Expression and Akt Signalling in Prostate Cancer. *PLoS ONE*, *8*(6), e65798.

Clarke, S. Rm., Barnden, M., Kurts, C., Carbone, F. R., Miller, J. F., & Heath, W. R. (2000). Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunology & Cell Biology*, *78*(2), 110–117.

Cohen, N., Shani, O., Raz, Y., Sharon, Y., Hoffman, D., Abramovitz, L., & Erez, N. (2017). Fibroblasts drive an immunosuppressive and growth-promoting microenvironment in breast cancer via secretion of Chitinase 3-like 1. *Oncogene*, *36*(31), 4457–4468.

Contassot, E., Wilmotte, R., Tenan, M., Belkouch, M.-C., Schnüriger, V., de Tribolet, N., Bourkhardt, K., & Dietrich, P.-Y. (2004). Arachidonylethanolamide Induces Apoptosis of Human Glioma Cells through Vanilloid Receptor-1. *Journal of Neuropathology & Experimental Neurology*, *63*(9), 956–963.

Coopman, K., Smith, L. D., Wright, K. L., & Ward, S. G. (2007). Temporal variation in CB2R levels following T lymphocyte activation: Evidence that cannabinoids modulate CXCL12-induced chemotaxis. *International Immunopharmacology*, *7*(3), 360–371.

Cortes, J., Rugo, H. S., Cescon, D. W., Im, S.-A., Yusof, M. M., Gallardo, C., Lipatov, O., Barrios, C. H., Perez-Garcia, J., Iwata, H., Masuda, N., Torregroza Otero, M., Gokmen, E., Loi, S., Guo, Z., Zhou, X., Karantza, V., Pan, W., & Schmid, P. (2022). Pembrolizumab plus Chemotherapy in Advanced Triple-Negative Breast Cancer. *New England Journal of Medicine*, *387*(3), 217–226.

Costa, A., Kieffer, Y., Scholer-Dahirel, A., Pelon, F., Bourachot, B., Cardon, M., Sirven, P., Magagna, I., Fuhrmann, L., Bernard, C., Bonneau, C., Kondratova, M., Kuperstein, I., Zinovyev, A., Givel, A.-M., Parrini, M.-C., Soumelis, V., Vincent-Salomon, A., & Mechta-Grigoriou, F. (2018). Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer. *Cancer Cell*, *33*(3), 463-479.e10.

Cremasco, V., Astarita, J. L., Grauel, A. L., Keerthivasan, S., MacIsaac, K., Woodruff, M. C., Wu, M., Spel, L., Santoro, S., Amoozgar, Z., Laszewski, T., Migoni, S. C., Knoblich, K., Fletcher, A. L., LaFleur, M., Wucherpfennig, K. W., Pure, E., Dranoff, G., Carroll, M. C., & Turley, S. J. (2018). FAP Delineates Heterogeneous and Functionally Divergent Stromal Cells in Immune-Excluded Breast Tumors. *Cancer Immunology Research*, *6*(12), 1472–1485.

Da Silva, F. C., Brandão, D. C., Ferreira, E. A., Siqueira, R. P., Ferreira, H. S. V., Da Silva Filho, A. A., & Araújo, T. G. (2023). Tailoring Potential Natural Compounds for the Treatment of Luminal Breast Cancer. *Pharmaceuticals*, *16*(10), 1466.

Dada, S., Ellis, S. L. S., Wood, C., Nohara, L. L., Dreier, C., Garcia, N. H., Saranchova, I., Munro, L., Pfeifer, C. G., Eyford, B. A., Kari, S., Garrovillas, E., Caspani, G., Al Haddad, E., Gray, P. W., Morova, T., Lack, N. A., Andersen, R. J., Tjoelker, L., & Jefferies, W. A. (2023). Specific cannabinoids revive adaptive immunity by reversing immune evasion mechanisms in metastatic tumours. *Frontiers in Immunology*, *13*.

Darling, V. R., Hauke, R. J., Tarantolo, S., & Agrawal, D. K. (2015). Immunological effects and therapeutic role of C5a in cancer. *Expert Review of Clinical Immunology*, *11*(2), 255–263.

Dawson, C. A., Pal, B., Vaillant, F., Gandolfo, L. C., Liu, Z., Bleriot, C., Ginhoux, F., Smyth, G. K., Lindeman, G. J., Mueller, S. N., Rios, A. C., & Visvader, J. E. (2020). Tissue-resident ductal macrophages survey the mammary epithelium and facilitate tissue remodelling. *Nature Cell Biology*, *22*(5), 546–558.

De Petrocellis, L., Ligresti, A., Schiano Moriello, A., Iappelli, M., Verde, R., Stott, C. G., Cristino, L., Orlando, P., & Di Marzo, V. (2013). Non-THC cannabinoids inhibit prostate carcinoma growth *in vitro* and *in vivo*: pro-apoptotic effects and underlying mechanisms. *British Journal of Pharmacology*, *168*(1), 79–102.

De Visser, K. E., & Joyce, J. A. (2023). The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. *Cancer Cell*, *41*(3), 374–403.

Delirezh, N., Moazzeni, S. M., Shokri, F., Shokrgozar, M. A., Morteza Atri, M., & Karbassian, H. (2012). In vitro analysis of T cell responses induced by breast tumor cell lysate pulsed with autologous dendritic cells. *Advances in Bioscience and Biotechnology*, *03*(02), 126–136.

DeNardo, D. G., & Coussens, L. M. (2007). Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Research*, *9*(4), 212.

Deng, L., Ng, L., Ozawa, T., & Stella, N. (2017). Quantitative Analyses of Synergistic Responses between Cannabidiol and DNA-Damaging Agents on the Proliferation and Viability of Glioblastoma and Neural Progenitor Cells in Culture. *The Journal of Pharmacology and Experimental Therapeutics*, *360*(1), 215–224.

Denkert, C., von Minckwitz, G., Darb-Esfahani, S., Lederer, B., Heppner, B. I., Weber, K. E., Budczies, J., Huober, J., Klauschen, F., Furlanetto, J., Schmitt, W. D., Blohmer, J.-U., Karn, T., Pfitzner, B. M., Kümmel, S., Engels, K., Schneeweiss, A., Hartmann, A., Noske, A., … Loibl, S. (2018). Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *The Lancet Oncology*, *19*(1), 40–50.

Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S., & Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Molecular Pharmacology*, *34*(5), 605–613.

Devane, W. A., Hanuš, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., & Mechoulam, R. (1992). Isolation and Structure of a Brain Constituent That Binds to the Cannabinoid Receptor. *Science*, *258*(5090), 1946–1949.

Dhital, S., Stokes, J. V., Park, N., Seo, K. S., & Kaplan, B. L. F. (2017). Cannabidiol (CBD) induces functional Tregs in response to low-level T cell activation. *Cellular Immunology*, *312*, 25–34.

Di Sabatino, A., Battista, N., Biancheri, P., Rapino, C., Rovedatti, L., Astarita, G., Vanoli, A., Dainese, E., Guerci, M., Piomelli, D., Pender, S. L. F., MacDonald, T. T., Maccarrone,

M., & Corazza, G. R. (2011). The endogenous cannabinoid system in the gut of patients with inflammatory bowel disease. *Mucosal Immunology*, *4*(5), 574–583.

Dieci, M. V., Radosevic-Robin, N., Fineberg, S., van den Eynden, G., Ternes, N., Penault-Llorca, F., Pruneri, G., D'Alfonso, T. M., Demaria, S., Castaneda, C., Sanchez, J., Badve, S., Michiels, S., Bossuyt, V., Rojo, F., Singh, B., Nielsen, T., Viale, G., Kim, S.-R., … Salgado, R. (2018). Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess TILs in residual disease after neoadjuvant therapy and in carcinoma in situ: A report of the International Immuno-Oncology Biomarker Working Group on Breast Cancer. *Seminars in Cancer Biology*, *52*, 16–25.

Do, Y., McKallip, R. J., Nagarkatti, M., & Nagarkatti, P. S. (2004). Activation through Cannabinoid Receptors 1 and 2 on Dendritic Cells Triggers NF-κB-Dependent Apoptosis: Novel Role for Endogenous and Exogenous Cannabinoids in Immunoregulation. *The Journal of Immunology*, *173*(4), 2373–2382.

Donati, K., Sépult, C., Rocks, N., Blacher, S., Gérard, C., Noel, A., & Cataldo, D. (2017). Neutrophil-Derived Interleukin 16 in Premetastatic Lungs Promotes Breast Tumor Cell Seeding. *Cancer Growth and Metastasis*, *10*, 117906441773851.

Duchemann, B., Naigeon, M., Auclin, E., Ferrara, R., Cassard, L., Jouniaux, J.-M., Boselli, L., Grivel, J., Desnoyer, A., Danlos, F.-X., Mezquita, L., Caramella, C., Marabelle, A., Besse, B., & Chaput, N. (2022). CD8 + PD-1 + to CD4 + PD-1 + ratio (PERLS) is associated with prognosis of patients with advanced NSCLC treated with PD-(L)1 blockers. *Journal for ImmunoTherapy of Cancer*, *10*(2), e004012.

Duerr, G. D., Heinemann, J. C., Suchan, G., Kolobara, E., Wenzel, D., Geisen, C., Matthey, M., Passe-Tietjen, K., Mahmud, W., Ghanem, A., Tiemann, K., Alferink, J., Burgdorf, S., Buchalla, R., Zimmer, A., Lutz, B., Welz, A., Fleischmann, B. K., & Dewald, O. (2014). The endocannabinoid-CB2 receptor axis protects the ischemic heart at the early stage of cardiomyopathy. *Basic Research in Cardiology*, *109*(4), 425.

Elbaz, M., Ahirwar, D., Ravi, J., Nasser, M. W., & Ganju, R. K. (2017). Novel role of cannabinoid receptor 2 in inhibiting EGF/EGFR and IGF-I/IGF-IR pathways in breast cancer. *Oncotarget*, *8*(18), 29668–29678.

Elbaz, M., Ahirwar, D., Xiaoli, Z., Zhou, X., Lustberg, M., Nasser, M. W., Shilo, K., & Ganju, R. K. (2018). TRPV2 is a novel biomarker and therapeutic target in triple negative breast cancer. *Oncotarget*, *9*(71), 33459–33470.

Elbaz, M., Nasser, M. W., Ravi, J., Wani, N. A., Ahirwar, D. K., Zhao, H., Oghumu, S., Satoskar, A. R., Shilo, K., Carson, W. E., & Ganju, R. K. (2015). Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: Novel anti-tumor mechanisms of Cannabidiol in breast cancer. *Molecular Oncology*, *9*(4), 906–919.

Elston, C. W., & Ellis, I. O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*, *19*(5), 403–410.

Erler, J. T., Bennewith, K. L., Cox, T. R., Lang, G., Bird, D., Koong, A., Le, Q.-T., & Giaccia, A. J. (2009). Hypoxia-Induced Lysyl Oxidase Is a Critical Mediator of Bone Marrow Cell Recruitment to Form the Premetastatic Niche. *Cancer Cell*, *15*(1), 35–44.

Faghih, Z., Erfani, N., Haghshenas, M. R., Safaei, A., Talei, A.-R., & Ghaderi, A. (2014). Immune profiles of CD4+ lymphocyte subsets in breast cancer tumor draining lymph nodes. *Immunology Letters*, *158*(1–2), 57–65.

Falcinelli, S. D., Cooper-Volkheimer, A. D., Semenova, L., Wu, E., Richardson, A., Ashokkumar, M., Margolis, D. M., Archin, N. M., Rudin, C. D., Murdoch, D., & Browne, E. P. (2022). *Impact of cannabis use on immune cell populations and the viral reservoir in people with HIV on suppressive antiretroviral therapy*.

Fang, J., Lu, Y., Zheng, J., Jiang, X., Shen, H., Shang, X., Lu, Y., & Fu, P. (2023). Exploring the crosstalk between endothelial cells, immune cells, and immune checkpoints in the tumor microenvironment: new insights and therapeutic implications. *Cell Death & Disease*, *14*(9), 586.

Fang, Y., Wang, L., Wan, C., Sun, Y., Van der Jeught, K., Zhou, Z., Dong, T., So, K. M., Yu, T., Li, Y., Eyvani, H., Colter, A. B., Dong, E., Cao, S., Wang, J., Schneider, B. P., Sandusky, G. E., Liu, Y., Zhang, C., … Zhang, X. (2021). MAL2 drives immune evasion in breast cancer by suppressing tumor antigen presentation. *Journal of Clinical Investigation*, *131*(1).

Farmer, P., Bonnefoi, H., Anderle, P., Cameron, D., Wirapati, P., Becette, V., André, S., Piccart, M., Campone, M., Brain, E., MacGrogan, G., Petit, T., Jassem, J., Bibeau, F., Blot, E., Bogaerts, J., Aguet, M., Bergh, J., Iggo, R., & Delorenzi, M. (2009). A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nature Medicine*, *15*(1), 68–74.

Felder, C. C., Joyce, K. E., Briley, E. M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A. L., & Mitchell, R. L. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Molecular Pharmacology*, *48*(3), 443–450.

Feng, D., Pu, D., Ren, J., Liu, M., Zhang, Z., Liu, Z., & Li, J. (2024). CD8+ T-cell exhaustion: Impediment to triple-negative breast cancer (TNBC) immunotherapy. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1879*(6), 189193.

Ferrini, M. E., Hong, S., Stierle, A., Stierle, D., Stella, N., Roberts, K., & Jaffar, Z. (2017). CB2 receptors regulate natural killer cells that limit allergic airway inflammation in a murine model of asthma. *Allergy*, *72*(6), 937–947.

Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S., Omeroglu, A., Hallett, M., & Park, M. (2008). Stromal gene expression predicts clinical outcome in breast cancer. *Nature Medicine*, *14*(5), 518–527.

Fracol, M., Datta, J., Lowenfeld, L., Xu, S., Zhang, P. J., Fisher, C. S., & Czerniecki, B. J. (2017). Loss of Anti-HER-3 CD4+ T-Helper Type 1 Immunity Occurs in Breast Tumorigenesis and is Negatively Associated with Outcomes. *Annals of Surgical Oncology*, *24*(2), 407–417.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., LE Fur, G., & Casellas, P. (1995). Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *European Journal of Biochemistry*, *232*(1), 54–61.

Galve-Roperh, I., Sánchez, C., Cortés, M. L., del Pulgar, T. G., Izquierdo, M., & Guzmán, M. (2000). Anti-tumoral action of cannabinoids: Involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nature Medicine*, *6*(3), 313–319.

Gao, M., Shi, J., Xiao, X., Yao, Y., Chen, X., Wang, B., & Zhang, J. (2024). PD-1 regulation in immune homeostasis and immunotherapy. *Cancer Letters*, *588*, 216726.

Gaoni, Y., & Mechoulam, R. (1964). Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *Journal of the American Chemical Society*, *86*(8), 1646–1647.

Garai, J., Dutil, J., Cress, D. W., Seewaldt, V., Teer, J. K., Fejerman, L., Miele, L., & Zabaleta, J. (2021). Abstract 2729: Cell infiltration prediction of breast cancer tissues identifies basophils as potentially important immune cells in the tumor microenvironment. *Cancer Research*, *81*(13_Supplement), 2729–2729.

Garaud, S., Zayakin, P., Buisseret, L., Rulle, U., Silina, K., de Wind, A., Van den Eyden, G., Larsimont, D., Willard-Gallo, K., & Linē, A. (2018). Antigen Specificity and Clinical Significance of IgG and IgA Autoantibodies Produced in situ by Tumor-Infiltrating B Cells in Breast Cancer. *Frontiers in Immunology*, *9*.

Gatti-Mays, M. E., Balko, J. M., Gameiro, S. R., Bear, H. D., Prabhakaran, S., Fukui, J., Disis, M. L., Nanda, R., Gulley, J. L., Kalinsky, K., Abdul Sater, H., Sparano, J. A., Cescon, D., Page, D. B., McArthur, H., Adams, S., & Mittendorf, E. A. (2019). If we build it they will come: targeting the immune response to breast cancer. *Npj Breast Cancer*, *5*(1), 37.

Gebremeskel, S., Clattenburg, D. R., Slauenwhite, D., Lobert, L., & Johnston, B. (2015). Natural killer T cell activation overcomes immunosuppression to enhance clearance of postsurgical breast cancer metastasis in mice. *OncoImmunology*, *4*(3), e995562.

Giaquinto, A. N., Sung, H., Newman, L. A., Freedman, R. A., Smith, R. A., Star, J., Jemal, A., & Siegel, R. L. (2024). Breast cancer statistics 2024. *CA: A Cancer Journal for Clinicians*, *74*(6), 477–495.

Gjerstorff, M., Benoit, V., Laenkholm, A.-V., Nielsen, O., Johansen, L., & Ditzel, H. (2006). Identification of genes with altered expression in medullary breast cancer vs. ductal breast cancer and normal breast epithelia. *International Journal of Oncology*.

Glodde, N., Jakobs, M., Bald, T., Tüting, T., & Gaffal, E. (2015). Differential role of cannabinoids in the pathogenesis of skin cancer. *Life Sciences*, *138*, 35–40.

Go, Y. Y., Kim, S. R., Kim, D. Y., Chae, S.-W., & Song, J.-J. (2020). Cannabidiol enhances cytotoxicity of anti-cancer drugs in human head and neck squamous cell carcinoma. *Scientific Reports*, *10*(1), 20622.

Gokoh, M., Kishimoto, S., Oka, S., Metani, Y., & Sugiura, T. (2005). 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, enhances the adhesion of HL-60 cells

differentiated into macrophage-like cells and human peripheral blood monocytes. *FEBS Letters*, *579*(28), 6473–6478.

Gokoh, M., Kishimoto, S., Oka, S., & Sugiura, T. (2007). 2-Arachidonoylglycerol Enhances the Phagocytosis of Opsonized Zymosan by HL-60 Cells Differentiated into Macrophage-Like Cells. *Biological and Pharmaceutical Bulletin*, *30*(7), 1199–1205.

Gopinath, A., Mackie, P. M., Phan, L. T., Mirabel, R., Smith, A. R., Miller, E., Franks, S., Syed, O., Riaz, T., Law, B. K., Urs, N., & Khoshbouei, H. (2023). Who Knew? Dopamine Transporter Activity Is Critical in Innate and Adaptive Immune Responses. *Cells*, *12*(2), 269.

Graham, E. S., Angel, C. E., Schwarcz, L. E., Dunbar, P. R., & Glass, M. (2010). Detailed Characterisation of CB2 Receptor Protein Expression in Peripheral Blood Immune Cells from Healthy Human Volunteers Using Flow Cytometry. *International Journal of Immunopathology and Pharmacology*, *23*(1), 25–34.

Grimaldi, C., Pisanti, S., Laezza, C., Malfitano, A. M., Santoro, A., Vitale, M., Caruso, M. G., Notarnicola, M., Iacuzzo, I., Portella, G., Di Marzo, V., & Bifulco, M. (2006). Anandamide inhibits adhesion and migration of breast cancer cells. *Experimental Cell Research*, *312*(4), 363–373.

Grimison, P., Mersiades, A., Kirby, A., Lintzeris, N., Morton, R., Haber, P., Olver, I., Walsh, A., McGregor, I., Cheung, Y., Tognela, A., Hahn, C., Briscoe, K., Aghmesheh, M., Fox, P., Abdi, E., Clarke, S., Della-Fiorentina, S., Shannon, J., … Stockler, M. (2020). Oral THC:CBD cannabis extract for refractory chemotherapy-induced nausea and vomiting: a randomised, placebo-controlled, phase II crossover trial. *Annals of Oncology*, *31*(11), 1553–1560.

Grover, P., Goel, P. N., & Greene, M. I. (2021). Regulatory T Cells: Regulation of Identity and Function. *Frontiers in Immunology*, *12*.

Gruber, T., Robatel, S., Kremenovic, M., Bäriswyl, L., Gertsch, J., & Schenk, M. (2021). Cannabinoid Receptor Type-2 in B Cells Is Associated with Tumor Immunity in Melanoma. *Cancers*, *13*(8), 1934.

Guida, M., Ligresti, A., De Filippis, D., D'Amico, A., Petrosino, S., Cipriano, M., Bifulco, G., Simonetti, S., Orlando, P., Insabato, L., Nappi, C., Di Spiezio Sardo, A., Di Marzo, V., & Iuvone, T. (2010). The Levels of the Endocannabinoid Receptor CB2 and Its Ligand 2-Arachidonoylglycerol Are Elevated in Endometrial Carcinoma. *Endocrinology*, *151*(3), 921–928.

Guo, Y., Hu, Y., Huang, D., Yang, J., Fu, S., Deng, X., Long, J., Wang, J., & Wang, Y. (2025). Dual roles of GM-CSF in breast cancer: Immunomodulation and therapeutic implications. *Critical Reviews in Oncology/Hematology*, *214*, 104804.

Guzmán, M., Duarte, M. J., Blázquez, C., Ravina, J., Rosa, M. C., Galve-Roperh, I., Sánchez, C., Velasco, G., & González-Feria, L. (2006). A pilot clinical study of Δ9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. *British Journal of Cancer*, *95*(2), 197–203.

Gwak, J. M., Jang, M. H., Kim, D. Il, Seo, A. N., & Park, S. Y. (2015). Prognostic Value of Tumor-Associated Macrophages According to Histologic Locations and Hormone Receptor Status in Breast Cancer. *PLOS ONE*, *10*(4), e0125728.

Hanahan, D. (2022). Hallmarks of Cancer: New Dimensions. *Cancer Discovery*, *12*(1), 31–46.

Harbeck, N., Penault-Llorca, F., Cortes, J., Gnant, M., Houssami, N., Poortmans, P., Ruddy, K., Tsang, J., & Cardoso, F. (2019). Breast cancer. *Nature Reviews Disease Primers*, *5*(1), 66.

Hart, S., Fischer, O. M., & Ullrich, A. (2004). Cannabinoids Induce Cancer Cell Proliferation via Tumor Necrosis Factor α-Converting Enzyme (TACE/ADAM17)-Mediated Transactivation of the Epidermal Growth Factor Receptor. *Cancer Research*, *64*(6), 1943–1950.

Hasenoehrl, C., Feuersinger, D., Sturm, E. M., Bärnthaler, T., Heitzer, E., Graf, R., Grill, M., Pichler, M., Beck, S., Butcher, L., Thomas, D., Ferreirós, N., Schuligoi, R., Schweiger, C., Haybaeck, J., & Schicho, R. (2018). G protein-coupled receptor GPR55 promotes colorectal cancer and has opposing effects to cannabinoid receptor 1. *International Journal of Cancer*, *142*(1), 121–132.

Hegde, V. L., Nagarkatti, P. S., & Nagarkatti, M. (2011). Role of Myeloid-Derived Suppressor Cells in Amelioration of Experimental Autoimmune Hepatitis Following Activation of TRPV1 Receptors by Cannabidiol. *PLoS ONE*, *6*(4), e18281.

Hegde, V., Nagarkatti, P., & Nagarkatti, M. (2010). Cannabidiol attenuates experimental autoimmune hepatitis by inducing accumulation of myeloid-derived suppressor cells (MDSCs) in liver (140.4). *The Journal of Immunology*, *184*(Supplement_1), 140.4-140.4.

Heng, T. S. P., Painter, M. W., Elpek, K., Lukacs-Kornek, V., Mauermann, N., Turley, S. J., Koller, D., Kim, F. S., Wagers, A. J., Asinovski, N., Davis, S., Fassett, M., Feuerer, M., Gray, D. H. D., Haxhinasto, S., Hill, J. A., Hyatt, G., Laplace, C., Leatherbee, K., … Kang, J. (2008). The Immunological Genome Project: networks of gene expression in immune cells. *Nature Immunology*, *9*(10), 1091–1094.

Hennersdorf, F., Florian, S., Jakob, A., Baumgärtner, K., Sonneck, K., Nordheim, A., Biedermann, T., Valent, P., & Bühring, H.-J. (2005). Identification of CD13, CD107a, and CD164 as novel basophil-activation markers and dissection of two response patterns in time kinetics of IgE-dependent upregulation. *Cell Research*, *15*(5), 325–335.

Henriquez, J. E., Bach, A. P., Matos-Fernandez, K. M., Crawford, R. B., & Kaminski, N. E. (2020). Δ9-Tetrahydrocannabinol (THC) Impairs CD8+ T Cell-Mediated Activation of Astrocytes. *Journal of Neuroimmune Pharmacology*, *15*(4), 863–874.

Hinz, B., & Ramer, R. (2022). Cannabinoids as anticancer drugs: current status of preclinical research. *British Journal of Cancer*, *127*(1), 1–13.

Hinz, B., Ramer, R., Eichele, K., Weinzierl, U., & Brune, K. (2004). Up-Regulation of Cyclooxygenase-2 Expression Is Involved in R(+)-Methanandamide-Induced

Apoptotic Death of Human Neuroglioma Cells. *Molecular Pharmacology*, *66*(6), 1643–1651.

Hix, L. M., Shi, Y. H., Brutkiewicz, R. R., Stein, P. L., Wang, C.-R., & Zhang, M. (2011). CD1d-Expressing Breast Cancer Cells Modulate NKT Cell-Mediated Antitumor Immunity in a Murine Model of Breast Cancer Metastasis. *PLoS ONE*, *6*(6), e20702.

Hohmann, T., Grabiec, U., Ghadban, C., Feese, K., & Dehghani, F. (2017). The influence of biomechanical properties and cannabinoids on tumor invasion. *Cell Adhesion & Migration*, *11*(1), 54–67.

Howard, F. M., Pearson, A. T., & Nanda, R. (2022). Clinical trials of immunotherapy in triple-negative breast cancer. *Breast Cancer Research and Treatment*, *195*(1), 1–15.

Howlett, A. C., & Fleming, R. M. (1984). Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Molecular Pharmacology*, *26*(3), 532–538.

Hua, T., Vemuri, K., Pu, M., Qu, L., Han, G. W., Wu, Y., Zhao, S., Shui, W., Li, S., Korde, A., Laprairie, R. B., Stahl, E. L., Ho, J.-H., Zvonok, N., Zhou, H., Kufareva, I., Wu, B., Zhao, Q., Hanson, M. A., … Liu, Z.-J. (2016). Crystal Structure of the Human Cannabinoid Receptor CB1. *Cell*, *167*(3), 750-762.e14.

Huang, Y., Ma, C., Zhang, Q., Ye, J., Wang, F., Zhang, Y., Hunborg, P., Varvares, M. A., Hoft, D. F., Hsueh, E. C., & Peng, G. (2015). CD4+ and CD8+ T cells have opposing roles in breast cancer progression and outcome. *Oncotarget*, *6*(19), 17462–17478.

Hughes, R., Qian, B.-Z., Rowan, C., Muthana, M., Keklikoglou, I., Olson, O. C., Tazzyman, S., Danson, S., Addison, C., Clemons, M., Gonzalez-Angulo, A. M., Joyce, J. A., De Palma, M., Pollard, J. W., & Lewis, C. E. (2015). Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy. *Cancer Research*, *75*(17), 3479–3491.

Ibsen, M. S., Connor, M., & Glass, M. (2017). Cannabinoid CB 1 and CB 2 Receptor Signaling and Bias. *Cannabis and Cannabinoid Research*, *2*(1), 48–60.

Iden, J. A., Raphael-Mizrahi, B., Awida, Z., Naim, A., Zyc, D., Liron, T., Kasher, M., Livshits, G., Vered, M., & Gabet, Y. (2023). The Anti-Tumorigenic Role of Cannabinoid Receptor 2 in Colon Cancer: A Study in Mice and Humans. *International Journal of Molecular Sciences*, *24*(4), 4060.

Iden, J. A., Raphael-Mizrahi, B., Naim, A., Kolomansky, A., Liron, T., Neumann, D., Vered, M., & Gabet, Y. (2023). The Anti-Tumorigenic Role of Cannabinoid Receptor 2 in Non-Melanoma Skin Cancer. *International Journal of Molecular Sciences*, *24*(9), 7773.

Ishigami, E., Sakakibara, M., Sakakibara, J., Masuda, T., Fujimoto, H., Hayama, S., Nagashima, T., Sangai, T., Nakagawa, A., Nakatani, Y., & Otsuka, M. (2019). Coexistence of regulatory B cells and regulatory T cells in tumor-infiltrating lymphocyte aggregates is a prognostic factor in patients with breast cancer. *Breast Cancer*, *26*(2), 180–189.

Itri, R., Junqueira, H. C., Mertins, O., & Baptista, M. S. (2014). Membrane changes under oxidative stress: the impact of oxidized lipids. *Biophysical Reviews*, *6*(1), 47–61.

Jacobsson, S. O. P., Rongård, E., Stridh, M., Tiger, G., & Fowler, C. J. (2000). Serum-dependent effects of tamoxifen and cannabinoids upon C6 glioma cell viability. *Biochemical Pharmacology*, *60*(12), 1807–1813.

Jamiyan, T., Kuroda, H., Yamaguchi, R., Nakazato, Y., Noda, S., Onozaki, M., Abe, A., & Hayashi, M. (2020). Prognostic impact of a tumor-infiltrating lymphocyte subtype in triple negative cancer of the breast. *Breast Cancer*, *27*(5), 880–892.

Jan, T.-R., Su, S.-T., Wu, H.-Y., & Liao, M.-H. (2007). Suppressive effects of cannabidiol on antigen-specific antibody production and functional activity of splenocytes in ovalbumin-sensitized BALB/c mice. *International Immunopharmacology*, *7*(6), 773–780.

Jaquish, A., Phung, E., Gong, X., Baldominos, P., Galvan-Pena, S., Bursulaya, I., Magill, I., Marina, E., Bertrand, K., Chambers, C., Muñoz-Rojas, A. R., Agudo, J., Mathis, D., Benoist, C., & Ramanan, D. (2024). *Expansion of mammary intraepithelial lymphocytes and intestinal inputs shape T cell dynamics in lactogenesis*.

Jenkins, E., Whitehead, T., Fellermeyer, M., Davis, S. J., & Sharma, S. (2023). The current state and future of T-cell exhaustion research. *Oxford Open Immunology*, *4*(1).

Jiang, Y., Li, Y., & Zhu, B. (2015). T-cell exhaustion in the tumor microenvironment. *Cell Death & Disease*, *6*(6), e1792–e1792.

Jorgovanovic, D., Song, M., Wang, L., & Zhang, Y. (2020). Roles of IFN-γ in tumor progression and regression: a review. *Biomarker Research*, *8*(1), 49.

Joseph, J., Niggemann, B., Zaenker, KurtS., & Entschladen, F. (2004). Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes. *Cancer Immunology, Immunotherapy*, *53*(8).

Jung, C. K., Kang, W. K., Park, J. M., Ahn, H. J., Kim, S. W., Taek Oh, S., & Choi, K. Y. (2013). Expression of the cannabinoid type I receptor and prognosis following surgery in colorectal cancer. *Oncology Letters*, *5*(3), 870–876.

Justo, B. L., & Jasiulionis, M. G. (2021). Characteristics of TIMP1, CD63, and β1-Integrin and the Functional Impact of Their Interaction in Cancer. *International Journal of Molecular Sciences*, *22*(17), 9319.

Kanemaru, H., Mizukami, Y., Kaneko, A., Kajihara, I., & Fukushima, S. (2022). A protocol for quantifying lymphocyte-mediated cytotoxicity using an impedance-based real-time cell analyzer. *STAR Protocols*, *3*(1), 101128.

Kaplan, R. N., Riba, R. D., Zacharoulis, S., Bramley, A. H., Vincent, L., Costa, C., MacDonald, D. D., Jin, D. K., Shido, K., Kerns, S. A., Zhu, Z., Hicklin, D., Wu, Y., Port, J. L., Altorki, N., Port, E. R., Ruggero, D., Shmelkov, S. V., Jensen, K. K., … Lyden, D. (2005). VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*, *438*(7069), 820–827.

Karmaus, P. W. F., Chen, W., Kaplan, B. L. F., & Kaminski, N. E. (2012). Δ9-Tetrahydrocannabinol Suppresses Cytotoxic T Lymphocyte Function Independent of CB1 and CB2, Disrupting Early Activation Events. *Journal of Neuroimmune Pharmacology*, *7*(4), 843–855.

Khan, S. U., Khan, M. U., Azhar Ud Din, M., Khan, I. M., Khan, M. I., Bungau, S., & Hassan, S. S. ul. (2023). Reprogramming tumor-associated macrophages as a unique approach to target tumor immunotherapy. *Frontiers in Immunology*, *14*.

Kim, H. R., Ha, S.-J., Hong, M. H., Heo, S. J., Koh, Y. W., Choi, E. C., Kim, E. K., Pyo, K. H., Jung, I., Seo, D., Choi, J., Cho, B. C., & Yoon, S. O. (2016). PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. *Scientific Reports*, *6*(1), 36956.

Klein, T. W., Newton, C., & Friedman, H. (1987). Inhibition of natural killer cell function by Marijuana components. *Journal of Toxicology and Environmental Health*, *20*(4), 321–332.

Klopfenstein, Q., Derangère, V., Arnould, L., Thibaudin, M., Limagne, E., Ghiringhelli, F., Truntzer, C., & Ladoire, S. (2021). Evaluation of tumor immune contexture among intrinsic molecular subtypes helps to predict outcome in early breast cancer. *Journal for ImmunoTherapy of Cancer*, *9*(6), e002036.

Kos, K., Salvagno, C., Wellenstein, M. D., Aslam, M. A., Meijer, D. A., Hau, C.-S., Vrijland, K., Kaldenbach, D., Raeven, E. A. M., Schmittnaegel, M., Ries, C. H., & de Visser, K. E. (2022). Tumor-associated macrophages promote intratumoral conversion of conventional CD4 + T cells into regulatory T cells via PD-1 signalling. *OncoImmunology*, *11*(1).

Kozela, E., Lev, N., Kaushansky, N., Eilam, R., Rimmerman, N., Levy, R., Ben-Nun, A., Juknat, A., & Vogel, Z. (2011). Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice. *British Journal of Pharmacology*, *163*(7), 1507–1519.

Krneta, T., Gillgrass, A., Chew, M., & Ashkar, A. A. (2016). The breast tumor microenvironment alters the phenotype and function of natural killer cells. *Cellular & Molecular Immunology*, *13*(5), 628–639.

Kryczek, I., Zou, L., Rodriguez, P., Zhu, G., Wei, S., Mottram, P., Brumlik, M., Cheng, P., Curiel, T., Myers, L., Lackner, A., Alvarez, X., Ochoa, A., Chen, L., & Zou, W. (2006). B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *The Journal of Experimental Medicine*, *203*(4), 871–881.

Kudou, M., Shiozaki, A., Yamazato, Y., Katsurahara, K., Kosuga, T., Shoda, K., Arita, T., Konishi, H., Komatsu, S., Kubota, T., Fujiwara, H., Okamoto, K., Kishimoto, M., Konishi, E., Marunaka, Y., & Otsuji, E. (2019). The expression and role of TRPV2 in esophageal squamous cell carcinoma. *Scientific Reports*, *9*(1), 16055.

Kuroda, H., Jamiyan, T., Yamaguchi, R., Kakumoto, A., Abe, A., Harada, O., Enkhbat, B., & Masunaga, A. (2021). Prognostic value of tumor-infiltrating B lymphocytes and plasma cells in triple-negative breast cancer. *Breast Cancer*, *28*(4), 904–914.

Kurozumi, S., Matsumoto, H., Kurosumi, M., Inoue, K., Fujii, T., Horiguchi, J., Shirabe, K., Oyama, T., & Kuwano, H. (2019). Prognostic significance of tumour-infiltrating lymphocytes for oestrogen receptor-negative breast cancer without lymph node metastasis. *Oncology Letters*.

Laezza, C., D'Alessandro, A., Paladino, S., Maria Malfitano, A., Chiara Proto, M., Gazzerro, P., Pisanti, S., Santoro, A., Ciaglia, E., & Bifulco, M. (2012). Anandamide inhibits the Wnt/β-catenin signalling pathway in human breast cancer MDA MB 231 cells. *European Journal of Cancer*, *48*(16), 3112–3122.

Laezza, C., Pisanti, S., Crescenzi, E., & Bifulco, M. (2006). Anandamide inhibits Cdk2 and activates Chk1 leading to cell cycle arrest in human breast cancer cells. *FEBS Letters*, *580*(26), 6076–6082.

Laezza, C., Pisanti, S., Malfitano, A. M., & Bifulco, M. (2008). The anandamide analog, Met-F-AEA, controls human breast cancer cell migration via the RHOA/RHO kinase signaling pathway. *Endocrine Related Cancer*, *15*(4), 965–974.

Law, A. M. K., Chen, J., Colino-Sanguino, Y., Fuente, L. R. de la, Fang, G., Grimes, S. M., Lu, H., Huang, R. J., Boyle, S. T., Venhuizen, J., Castillo, L., Tavakoli, J., Skhinas, J. N., Millar, E. K. A., Beretov, J., Rossello, F. J., Tipper, J. L., Ormandy, C. J., Samuel, M. S., … Gallego-Ortega, D. (2022). ALTEN: A High-Fidelity Primary Tissue-Engineering Platform to Assess Cellular Responses Ex Vivo. *Advanced Science*, *9*(21).

Lee, H. J., Park, I. A., Song, I. H., Shin, S.-J., Kim, J. Y., Yu, J. H., & Gong, G. (2016). Tertiary lymphoid structures: prognostic significance and relationship with tumour-infiltrating lymphocytes in triple-negative breast cancer. *Journal of Clinical Pathology*, *69*(5), 422–430.

Lee, J. S., Yost, S. E., & Yuan, Y. (2020). Neoadjuvant Treatment for Triple Negative Breast Cancer: Recent Progresses and Challenges. *Cancers*, *12*(6), 1404.

Lee, S. F., Newton, C., Widen, R., Friedman, H., & Klein, T. W. (2001a). Differential expression of cannabinoid CB2 receptor mRNA in mouse immune cell subpopulations and following B cell stimulation. *European Journal of Pharmacology*, *423*(2–3), 235–241.

Lee, S. F., Newton, C., Widen, R., Friedman, H., & Klein, T. W. (2001b). Downregulation of Cannabinoid Receptor 2 (CB2) Messenger RNA Expression During In Vitro Stimulation of Murine Splenocytes with Lipopolysaccharide. In *Neuroimmune Circuits, Drugs of Abuse, and Infectious Diseases* (pp. 223–228). Kluwer Academic Publishers.

Leek, R. D., Hunt, N. C., Landers, R. J., Lewis, C. E., Royds, J. A., & Harris, A. L. (2000). Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *The Journal of Pathology*, *190*(4), 430–436.

Lehmann, B. D., Jovanović, B., Chen, X., Estrada, M. V., Johnson, K. N., Shyr, Y., Moses, H. L., Sanders, M. E., & Pietenpol, J. A. (2016). Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. *PLOS ONE*, *11*(6), e0157368.

Leone, P., Malerba, E., Susca, N., Favoino, E., Perosa, F., Brunori, G., Prete, M., & Racanelli, V. (2024). Endothelial cells in tumor microenvironment: insights and perspectives. *Frontiers in Immunology*, *15*.

Lerner, E. C., Woroniecka, K. I., D'Anniballe, V. M., Wilkinson, D. S., Mohan, A. A., Lorrey, S. J., Waibl-Polania, J., Wachsmuth, L. P., Miggelbrink, A. M., Jackson, J. D., Cui, X., Raj, J. A., Tomaszewski, W. H., Cook, S. L., Sampson, J. H., Patel, A. P., Khasraw, M., Gunn,

M. D., & Fecci, P. E. (2023). CD8+ T cells maintain killing of MHC-I-negative tumor cells through the NKG2D–NKG2DL axis. *Nature Cancer*, *4*(9), 1258–1272.

Li, D., Ji, H., Niu, X., Yin, L., Wang, Y., Gu, Y., Wang, J., Zhou, X., Zhang, H., & Zhang, Q. (2020). Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer. *Cancer Science*, *111*(1), 47–58.

Li, L., Zhang, F., Liu, Z., & Fan, Z. (2023). Immunotherapy for Triple-Negative Breast Cancer: Combination Strategies to Improve Outcome. *Cancers*, *15*(1), 321.

Li, M., Knight, D. A., Snyder, L. A., Smyth, M. J., & Stewart, T. J. (2013). A role for CCL2 in both tumor progression and immunosurveillance. *OncoImmunology*, *2*(7), e25474.

Li, M., Quintana, A., Alberts, E., Hung, M. S., Boulat, V., Ripoll, M. M., & Grigoriadis, A. (2023). B Cells in Breast Cancer Pathology. *Cancers*, *15*(5), 1517.

Li, Q., Lao, X., Pan, Q., Ning, N., Yet, J., Xu, Y., Li, S., & Chang, A. E. (2011). Adoptive Transfer of Tumor Reactive B Cells Confers Host T-Cell Immunity and Tumor Regression. *Clinical Cancer Research*, *17*(15), 4987–4995.

Li, X., Hua, T., Vemuri, K., Ho, J.-H., Wu, Y., Wu, L., Popov, P., Benchama, O., Zvonok, N., Locke, K., Qu, L., Han, G. W., Iyer, M. R., Cinar, R., Coffey, N. J., Wang, J., Wu, M., Katritch, V., Zhao, S., … Liu, Z.-J. (2019). Crystal Structure of the Human Cannabinoid Receptor CB2. *Cell*, *176*(3), 459-467.e13.

Liang, H., Huang, J., Li, H., He, W., Ao, X., Xie, Z., Chen, Y., Lv, Z., zhang, L., Zhong, Y., Tan, X., Han, G., Zhou, J., Qiu, N., Jiang, M., Xia, H., Zhan, Y., Jiao, L., Ma, J., … Zhang, X. (2025). Spatial proximity of CD8+ T cells to tumor cells predicts neoadjuvant therapy efficacy in breast cancer. *Npj Breast Cancer*, *11*(1), 13.

Liang, S., Tran, E., Du, X., Dong, J., Sudholz, H., Chen, H., Qu, Z., Huntington, N. D., Babon, J. J., Kershaw, N. J., Zhang, Z.-Y., Baell, J. B., Wiede, F., & Tiganis, T. (2023). A small molecule inhibitor of PTP1B and PTPN2 enhances T cell anti-tumor immunity. *Nature Communications*, *14*(1), 4524.

Liao, D., Luo, Y., Markowitz, D., Xiang, R., & Reisfeld, R. A. (2009). Cancer Associated Fibroblasts Promote Tumor Growth and Metastasis by Modulating the Tumor Immune Microenvironment in a 4T1 Murine Breast Cancer Model. *PLoS ONE*, *4*(11), e7965.

Lin, E. Y., Li, J.-F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., Qian, H., Xue, X., & Pollard, J. W. (2006). Macrophages Regulate the Angiogenic Switch in a Mouse Model of Breast Cancer. *Cancer Research*, *66*(23), 11238–11246.

Liu, C., Somasundaram, A., Manne, S., Gocher, A. M., Szymczak-Workman, A. L., Vignali, K. M., Scott, E. N., Normolle, D. P., John Wherry, E., Lipson, E. J., Ferris, R. L., Bruno, T. C., Workman, C. J., & Vignali, D. A. A. (2020). Neuropilin-1 is a T cell memory checkpoint limiting long-term antitumor immunity. *Nature Immunology*, *21*(9), 1010–1021.

Liu, J., Zhang, N., Li, Q., Zhang, W., Ke, F., Leng, Q., Wang, H., Chen, J., & Wang, H. (2011). Tumor-Associated Macrophages Recruit CCR6+ Regulatory T Cells and Promote the

Development of Colorectal Cancer via Enhancing CCL20 Production in Mice. *PLoS ONE*, *6*(4), e19495.

Liu, Q. -R., Pan, C. -H., Hishimoto, A., Li, C. -Y., Xi, Z. -X., Llorente-Berzal, A., Viveros, M. -P., Ishiguro, H., Arinami, T., Onaivi, E. S., & Uhl, G. R. (2009). Species differences in cannabinoid receptor 2 (*CNR2* gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. *Genes, Brain and Behavior*, *8*(5), 519–530.

Liu, S., Foulkes, W. D., Leung, S., Gao, D., Lau, S., Kos, Z., & Nielsen, T. O. (2014). Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. *Breast Cancer Research*, *16*(5), 432.

Loi, S., Drubay, D., Adams, S., Pruneri, G., Francis, P. A., Lacroix-Triki, M., Joensuu, H., Dieci, M. V., Badve, S., Demaria, S., Gray, R., Munzone, E., Lemonnier, J., Sotiriou, C., Piccart, M. J., Kellokumpu-Lehtinen, P.-L., Vingiani, A., Gray, K., Andre, F., … Michiels, S. (2019). Tumor-Infiltrating Lymphocytes and Prognosis: A Pooled Individual Patient Analysis of Early-Stage Triple-Negative Breast Cancers. *Journal of Clinical Oncology*, *37*(7), 559–569.

Loi, S., Salgado, R., Schmid, P., Cortes, J., Cescon, D. W., Winer, E. P., Toppmeyer, D. L., Rugo, H. S., De Laurentiis, M., Nanda, R., Iwata, H., Awada, A., Tan, A. R., Sun, Y., Karantza, V., Wang, A., Huang, L., Saadatpour, A., Cristescu, R., … Adams, S. (2023). Association Between Biomarkers and Clinical Outcomes of Pembrolizumab Monotherapy in Patients With Metastatic Triple-Negative Breast Cancer: KEYNOTE-086 Exploratory Analysis. *JCO Precision Oncology*, *7*.

Loi, S., Sirtaine, N., Piette, F., Salgado, R., Viale, G., Van Eenoo, F., Rouas, G., Francis, P., Crown, J. P. A., Hitre, E., de Azambuja, E., Quinaux, E., Di Leo, A., Michiels, S., Piccart, M. J., & Sotiriou, C. (2013). Prognostic and Predictive Value of Tumor-Infiltrating Lymphocytes in a Phase III Randomized Adjuvant Breast Cancer Trial in Node-Positive Breast Cancer Comparing the Addition of Docetaxel to Doxorubicin With Doxorubicin-Based Chemotherapy: BIG 02-98. *Journal of Clinical Oncology*, *31*(7), 860–867.

Loi, S., Winer, E., Lipatov, O., Im, S.-A., Goncalves, A., Cortes, J., Lee, K. S., Schmid, P., Testa, L., Witzel, I., Ohtani, S., Turner, N., Zambelli, S., Harbeck, N., Andre, F., Dent, R., Huang, L., Mejia, J., Karantza, V., & Salgado, R. (2020). Abstract PD5-03: Relationship between tumor-infiltrating lymphocytes (TILs) and outcomes in the KEYNOTE-119 study of pembrolizumab vs chemotherapy for previously treated metastatic triple-negative breast cancer (mTNBC). *Cancer Research*, *80*(4_Supplement), PD5-03-PD5-03.

Loibl, S., Schneeweiss, A., Huober, J., Braun, M., Rey, J., Blohmer, J.-U., Furlanetto, J., Zahm, D.-M., Hanusch, C., Thomalla, J., Jackisch, C., Staib, P., Link, T., Rhiem, K., Solbach, C., Fasching, P. A., Nekljudova, V., Denkert, C., & Untch, M. (2022). Neoadjuvant durvalumab improves survival in early triple-negative breast cancer independent of pathological complete response. *Annals of Oncology*, *33*(11), 1149–1158.

Lombard, C., Nagarkatti, M., & Nagarkatti, P. (2007). CB2 cannabinoid receptor agonist, JWH-015, triggers apoptosis in immune cells: Potential role for CB2-selective ligands as immunosuppressive agents. *Clinical Immunology*, *122*(3), 259–270.

Long, C., Xie, N., Shu, Y., Wu, Y., He, P., Zhou, Y., Xiang, Y., Gu, J., Yang, L., & Wang, Y. (2021). Knockout of the Cannabinoid Receptor 2 Gene Promotes Inflammation and Hepatic Stellate Cell Activation by Promoting A20/Nuclear Factor-κB (NF-κB) Expression in Mice with Carbon Tetrachloride-Induced Liver Fibrosis. *Medical Science Monitor*, *27*.

Lonsdale, J., Thomas, J., Salvatore, M., Phillips, R., Lo, E., Shad, S., Hasz, R., Walters, G., Garcia, F., Young, N., Foster, B., Moser, M., Karasik, E., Gillard, B., Ramsey, K., Sullivan, S., Bridge, J., Magazine, H., Syron, J., … Moore, H. F. (2013). The Genotype-Tissue Expression (GTEx) project. *Nature Genetics*, *45*(6), 580–585.

López, A., Aparicio, N., Pazos, M. R., Grande, M. T., Barreda-Manso, M. A., Benito-Cuesta, I., Vázquez, C., Amores, M., Ruiz-Pérez, G., García-García, E., Beatka, M., Tolón, R. M., Dittel, B. N., Hillard, C. J., & Romero, J. (2018). Cannabinoid CB2 receptors in the mouse brain: relevance for Alzheimer's disease. *Journal of Neuroinflammation*, *15*(1), 158.

López-Valero, I., Torres, S., Salazar-Roa, M., García-Taboada, E., Hernández-Tiedra, S., Guzmán, M., Sepúlveda, J. M., Velasco, G., & Lorente, M. (2018). Optimization of a preclinical therapy of cannabinoids in combination with temozolomide against glioma. *Biochemical Pharmacology*, *157*, 275–284.

Luo, C.-K., Chou, P.-H., Ng, S.-K., Lin, W.-Y., & Wei, T.-T. (2022). Cannabinoids orchestrate cross-talk between cancer cells and endothelial cells in colorectal cancer. *Cancer Gene Therapy*, *29*(5), 597–611.

Maccarrone, M., Bab, I., Bíró, T., Cabral, G. A., Dey, S. K., Di Marzo, V., Konje, J. C., Kunos, G., Mechoulam, R., Pacher, P., Sharkey, K. A., & Zimmer, A. (2015). Endocannabinoid signaling at the periphery: 50 years after THC. *Trends in Pharmacological Sciences*, *36*(5), 277–296.

Mackie, K., Lai, Y., Westenbroek, R., & Mitchell, R. (1995). Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *The Journal of Neuroscience*, *15*(10), 6552–6561.

Maddukuri, S., Patel, J., Diaz, D. A., Chen, K. L., Wysocka, M., Bax, C., Li, Y., Ravishankar, A., Grinnell, M., Zeidi, M., Reddy, N., Concha, J. S. S., Bashir, M. M., Okawa, J., White, B., & Werth, V. P. (2022). Cannabinoid type 2 receptor (CB2R) distribution in dermatomyositis skin and peripheral blood mononuclear cells (PBMCs) and in vivo effects of LenabasumTM. *Arthritis Research & Therapy*, *24*(1), 12.

Maekawa, Y., Minato, Y., Ishifune, C., Kurihara, T., Kitamura, A., Kojima, H., Yagita, H., Sakata-Yanagimoto, M., Saito, T., Taniuchi, I., Chiba, S., Sone, S., & Yasutomo, K. (2008). Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nature Immunology*, *9*(10), 1140–1147.

Maes, M., Rachayon, M., Jirakran, K., Sughondhabirom, A., Almulla, A. F., & Sodsai, P. (2023). *Role of T and B lymphocyte cannabinoid type 1 and 2 receptors in major depression and suicidal behaviors: effects of in vitro cannabidiol administration*.

Maestroni, G. J. M. (2004). The endogenous cannabinoid 2-arachidonoyl glycerol as in vivo chemoattractant for dendritic cells and adjuvant for Th1 response to a soluble protein. *The FASEB Journal*, *18*(15), 1914–1916.

Mahmoud, S. M. A., Lee, A. H. S., Paish, E. C., Macmillan, R. D., Ellis, I. O., & Green, A. R. (2012). The prognostic significance of B lymphocytes in invasive carcinoma of the breast. *Breast Cancer Research and Treatment*, *132*(2), 545–553.

Mahmoud, S. M. A., Paish, E. C., Powe, D. G., Macmillan, R. D., Grainge, M. J., Lee, A. H. S., Ellis, I. O., & Green, A. R. (2011). Tumor-Infiltrating CD8 + Lymphocytes Predict Clinical Outcome in Breast Cancer. *Journal of Clinical Oncology*, *29*(15), 1949–1955.

Mamessier, E., Pradel, L. C., Thibult, M.-L., Drevet, C., Zouine, A., Jacquemier, J., Houvenaeghel, G., Bertucci, F., Birnbaum, D., & Olive, D. (2013). Peripheral Blood NK Cells from Breast Cancer Patients Are Tumor-Induced Composite Subsets. *The Journal of Immunology*, *190*(5), 2424–2436.

Maresz, K., Pryce, G., Ponomarev, E. D., Marsicano, G., Croxford, J. L., Shriver, L. P., Ledent, C., Cheng, X., Carrier, E. J., Mann, M. K., Giovannoni, G., Pertwee, R. G., Yamamura, T., Buckley, N. E., Hillard, C. J., Lutz, B., Baker, D., & Dittel, B. N. (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nature Medicine*, *13*(4), 492–497.

Marone, G., Gambardella, A. R., Mattei, F., Mancini, J., Schiavoni, G., & Varricchi, G. (2020). Basophils in Tumor Microenvironment and Surroundings. In *Tumor Microenvironment* (pp. 21–34).

Martínez-Martínez, E., Gómez, I., Martín, P., Sánchez, A., Román, L., Tejerina, E., Bonilla, F., Merino, A. G., de Herreros, A. G., Provencio, M., & García, J. M. (2015). Cannabinoids receptor type 2, CB2, expression correlates with human colon cancer progression and predicts patient survival. *Oncoscience*, *2*(2), 131–141.

Martínez-Martínez, E., Martín-Ruiz, A., Martín, P., Calvo, V., Provencio, M., & García, J. M. (2016). CB2 cannabinoid receptor activation promotes colon cancer progression via AKT/GSK3β signaling pathway. *Oncotarget*, *7*(42), 68781–68791.

Marzęda, P., Wróblewska-Łuczka, P., Drozd, M., Florek-Łuszczki, M., Załuska-Ogryzek, K., & Łuszczki, J. J. (2022). Cannabidiol Interacts Antagonistically with Cisplatin and Additively with Mitoxantrone in Various Melanoma Cell Lines—An Isobolographic Analysis. *International Journal of Molecular Sciences*, *23*(12), 6752.

Massi, P., Fuzio, D., Viganò, D., Sacerdote, P., & Parolaro, D. (2000). Relative involvement of cannabinoid CB1 and CB2 receptors in the Δ9-tetrahydrocannabinol-induced inhibition of natural killer activity. *European Journal of Pharmacology*, *387*(3), 343–347.

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., & Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, *346*(6284), 561–564.

McKallip, R. J., Lombard, C., Fisher, M., Martin, B. R., Ryu, S., Grant, S., Nagarkatti, P. S., & Nagarkatti, M. (2002). Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood*, *100*(2), 627–634.

McKallip, R. J., Lombard, C., Martin, B. R., Nagarkatti, M., & Nagarkatti, P. S. (2002). Δ9-Tetrahydrocannabinol-Induced Apoptosis in the Thymus and Spleen as a Mechanism of Immunosuppression in Vitro and in Vivo. *The Journal of Pharmacology and Experimental Therapeutics*, *302*(2), 451–465.

McKallip, R. J., Nagarkatti, M., & Nagarkatti, P. S. (2005). Δ-9-Tetrahydrocannabinol Enhances Breast Cancer Growth and Metastasis by Suppression of the Antitumor Immune Response. *The Journal of Immunology*, *174*(6), 3281–3289.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., Pertwee, R. G., Griffin, G., Bayewitch, M., Barg, J., & Vogel, Z. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical Pharmacology*, *50*(1), 83–90.

Mechoulam, R., Hanuš, L. O., Pertwee, R., & Howlett, A. C. (2014). Early phytocannabinoid chemistry to endocannabinoids and beyond. *Nature Reviews Neuroscience*, *15*(11), 757–764.

Mechoulam, R., & Shvo, Y. (1963). Hashish—I. *Tetrahedron*, *19*(12), 2073–2078.

Mehta, A. K., Kadel, S., Townsend, M. G., Oliwa, M., & Guerriero, J. L. (2021). Macrophage Biology and Mechanisms of Immune Suppression in Breast Cancer. *Frontiers in Immunology*, *12*.

Ménard, S., Pupa, S. M., Campiglio, M., & Tagliabue, E. (2003). Biologic and therapeutic role of HER2 in cancer. *Oncogene*, *22*(42), 6570–6578.

Messalli, E. M., Grauso, F., Luise, R., Angelini, A., & Rossiello, R. (2014). Cannabinoid receptor type 1 immunoreactivity and disease severity in human epithelial ovarian tumors. *American Journal of Obstetrics and Gynecology*, *211*(3), 234.e1-234.e6.

Michalski, C. W., Oti, F. E., Erkan, M., Sauliunaite, D., Bergmann, F., Pacher, P., Batkai, S., Müller, M. W., Giese, N. A., Friess, H., & Kleeff, J. (2008). Cannabinoids in pancreatic cancer: Correlation with survival and pain. *International Journal of Cancer*, *122*(4), 742–750.

Miles, D., Gligorov, J., André, F., Cameron, D., Schneeweiss, A., Barrios, C., Xu, B., Wardley, A., Kaen, D., Andrade, L., Semiglazov, V., Reinisch, M., Patel, S., Patre, M., Morales, L., Patel, S. L., Kaul, M., Barata, T., O'Shaughnessy, J., … Tabane, K. (2021). Primary results from IMpassion131, a double-blind, placebo-controlled, randomised phase III trial of first-line paclitaxel with or without atezolizumab for unresectable locally advanced/metastatic triple-negative breast cancer. *Annals of Oncology*, *32*(8), 994–1004.

Mimeault, M., Pommery, N., Wattez, N., Bailly, C., & Hénichart, J. (2003). Anti-proliferative and apoptotic effects of anandamide in human prostatic cancer cell lines: Implication of epidermal growth factor receptor down-regulation and ceramide production. *The Prostate*, *56*(1), 1–12.

Miyato, H., Kitayama, J., Yamashita, H., Souma, D., Asakage, M., Yamada, J., & Nagawa, H. (2009). Pharmacological Synergism Between Cannabinoids and Paclitaxel in Gastric Cancer Cell Lines. *Journal of Surgical Research*, *155*(1), 40–47.

Mohammed, Z. M. A., Going, J. J., Edwards, J., Elsberger, B., & McMillan, D. C. (2013). The relationship between lymphocyte subsets and clinico-pathological determinants of survival in patients with primary operable invasive ductal breast cancer. *British Journal of Cancer*, *109*(6), 1676–1684.

Mohammed, Z. M., Going, J. J., Edwards, J., Elsberger, B., Doughty, J. C., & McMillan, D. C. (2012). The relationship between components of tumour inflammatory cell infiltrate and clinicopathological factors and survival in patients with primary operable invasive ductal breast cancer. *British Journal of Cancer*, *107*(5), 864–873.

Monaco, G., Lee, B., Xu, W., Mustafah, S., Hwang, Y. Y., Carré, C., Burdin, N., Visan, L., Ceccarelli, M., Poidinger, M., Zippelius, A., Pedro de Magalhães, J., & Larbi, A. (2019). RNA-Seq Signatures Normalized by mRNA Abundance Allow Absolute Deconvolution of Human Immune Cell Types. *Cell Reports*, *26*(6), 1627-1640.e7.

Montero, C., Campillo, N. E., Goya, P., & Páez, J. A. (2005). Homology models of the cannabinoid CB1 and CB2 receptors. A docking analysis study. *European Journal of Medicinal Chemistry*, *40*(1), 75–83.

Moreno, E., Cavic, M., Krivokuca, A., Casadó, V., & Canela, E. (2019). The Endocannabinoid System as a Target in Cancer Diseases: Are We There Yet? *Frontiers in Pharmacology*, *10*.

Moura, T., Laranjeira, P., Caramelo, O., Gil, A. M., & Paiva, A. (2025). Breast Cancer and Tumor Microenvironment: The Crucial Role of Immune Cells. *Current Oncology*, *32*(3), 143.

Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verástegui, E., & Zlotnik, A. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, *410*(6824), 50–56.

Munro, S., Thomas, K. L., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, *365*(6441), 61–65.

Munson, A. E., Harris, L. S., Friedman, M. A., Dewey, W. L., & Carchman, R. A. (1975). Antineoplastic Activity of Cannabinoids2. *JNCI: Journal of the National Cancer Institute*, *55*(3), 597–602.

Muntasell, A., Rojo, F., Servitja, S., Rubio-Perez, C., Cabo, M., Tamborero, D., Costa-García, M., Martínez-Garcia, M., Menéndez, S., Vazquez, I., Lluch, A., Gonzalez-Perez, A., Rovira, A., López-Botet, M., & Albanell, J. (2019). NK Cell Infiltrates and HLA Class I Expression in Primary HER2+ Breast Cancer Predict and Uncouple Pathological Response and Disease-free Survival. *Clinical Cancer Research*, *25*(5), 1535–1545.

Murdamoothoo, D., Sun, Z., Yilmaz, A., Riegel, G., Abou-Faycal, C., Deligne, C., Velazquez-Quesada, I., Erne, W., Nascimento, M., Mörgelin, M., Cremel, G., Paul, N., Carapito, R., Veber, R., Dumortier, H., Yuan, J., Midwood, K. S., Loustau, T., & Orend, G. (2021).

Tenascin-C immobilizes infiltrating T lymphocytes through CXCL12 promoting breast cancer progression. *EMBO Molecular Medicine*, *13*(6).

Nabissi, M., Morelli, M. B., Santoni, M., & Santoni, G. (2013). Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents. *Carcinogenesis*, *34*(1), 48–57.

Nederlof, I., Isaeva, O. I., de Graaf, M., Gielen, R. C. A. M., Bakker, N. A. M., Rolfes, A. L., Garner, H., Boeckx, B., Traets, J. J. H., Mandjes, I. A. M., de Maaker, M., van Brussel, T., Chelushkin, M., Champanhet, E., Lopez-Yurda, M., van de Vijver, K., van den Berg, J. G., Hofland, I., Klioueva, N., … Kok, M. (2024). Neoadjuvant nivolumab or nivolumab plus ipilimumab in early-stage triple-negative breast cancer: a phase 2 adaptive trial. *Nature Medicine*, *30*(11), 3223–3235.

Nent, E., Nozaki, C., Schmöle, A.-C., Otte, D., & Zimmer, A. (2019). CB2 receptor deletion on myeloid cells enhanced mechanical allodynia in a mouse model of neuropathic pain. *Scientific Reports*, *9*(1), 7468.

Nersesian, S., Schwartz, S. L., Grantham, S. R., MacLean, L. K., Lee, S. N., Pugh-Toole, M., & Boudreau, J. E. (2021). NK cell infiltration is associated with improved overall survival in solid cancers: A systematic review and meta-analysis. *Translational Oncology*, *14*(1), 100930.

Nieto-Velázquez, N. G., Torres-Ramos, Y. D., Muñoz-Sánchez, J. L., Espinosa-Godoy, L., Gómez-Cortés, S., Moreno, J., & Moreno-Eutimio, M. A. (2016). Altered Expression of Natural Cytotoxicity Receptors and NKG2D on Peripheral Blood NK Cell Subsets in Breast Cancer Patients. *Translational Oncology*, *9*(5), 384–391.

Obeagu, E. I., & Obeagu, G. U. (2024). Exploring neutrophil functionality in breast cancer progression: A review. *Medicine*, *103*(13), e37654.

Okamura, K., Nagayama, S., Tate, T., Chan, H. T., Kiyotani, K., & Nakamura, Y. (2022). Lymphocytes in tumor-draining lymph nodes co-cultured with autologous tumor cells for adoptive cell therapy. *Journal of Translational Medicine*, *20*(1), 241.

Olkhanud, P. B., Baatar, D., Bodogai, M., Hakim, F., Gress, R., Anderson, R. L., Deng, J., Xu, M., Briest, S., & Biragyn, A. (2009). Breast Cancer Lung Metastasis Requires Expression of Chemokine Receptor CCR4 and Regulatory T Cells. *Cancer Research*, *69*(14), 5996–6004.

Orditura, M., Galizia, G., Diana, A., Saccone, C., Cobellis, L., Ventriglia, J., Iovino, F., Romano, C., Morgillo, F., Mosca, L., Diadema, M. R., Lieto, E., Procaccini, E., De Vita, F., & Ciardiello, F. (2016). Neutrophil to lymphocyte ratio (NLR) for prediction of distant metastasis-free survival (DMFS) in early breast cancer: a propensity score-matched analysis. *ESMO Open*, *1*(2), e000038.

O'Reilly, D., Sendi, M. Al, & Kelly, C. M. (2021). Overview of recent advances in metastatic triple negative breast cancer. *World Journal of Clinical Oncology*, *12*(3), 164–182.

Orellana-Serradell, O., Poblete, C. E., Sánchez, C., Castellón, E. A., Gallegos, I., Huidobro, C., Llanos, M. N., & Contreras, H. R. (2015). Proapoptotic effect of endocannabinoids in prostate cancer cells. *Oncology Reports*, *33*(4), 1599–1608.

Oshi, M., Asaoka, M., Tokumaru, Y., Yan, L., Matsuyama, R., Ishikawa, T., Endo, I., & Takabe, K. (2020). CD8 T Cell Score as a Prognostic Biomarker for Triple Negative Breast Cancer. *International Journal of Molecular Sciences*, *21*(18), 6968.

Oshi, M., Newman, S., Tokumaru, Y., Yan, L., Matsuyama, R., Kalinski, P., Endo, I., & Takabe, K. (2020). Plasmacytoid Dendritic Cell (pDC) Infiltration Correlate with Tumor Infiltrating Lymphocytes, Cancer Immunity, and Better Survival in Triple Negative Breast Cancer (TNBC) More Strongly than Conventional Dendritic Cell (cDC). *Cancers*, *12*(11), 3342.

Ostapchuk, Y. O., Cetin, E. A., Perfilyeva, Y. V., Yilmaz, A., Skiba, Y. A., Chirkin, A. P., Omarbaeva, N. A., Talaeva, S. G., Belyaev, N. N., & Deniz, G. (2015). Peripheral blood NK cells expressing HLA-G, IL-10 and TGF-β in healthy donors and breast cancer patients. *Cellular Immunology*, *298*(1–2), 37–46.

Owen, K. L., Brockwell, N. K., & Parker, B. S. (2019). JAK-STAT Signaling: A Double-Edged Sword of Immune Regulation and Cancer Progression. *Cancers*, *11*(12), 2002.

Oz, M., Yang, K.-H. S., & Mahgoub, M. O. (2022). Effects of cannabinoids on ligand-gated ion channels. *Frontiers in Physiology*, *13*.

Pacher, P., Bátkai, S., & Kunos, G. (2006). The Endocannabinoid System as an Emerging Target of Pharmacotherapy. *Pharmacological Reviews*, *58*(3), 389–462.

Pacher, P., Kogan, N. M., & Mechoulam, R. (2020). Beyond THC and Endocannabinoids. *Annual Review of Pharmacology and Toxicology*, *60*(1), 637–659.

Paget, S. (1889). The distribution of secondary growths in cancer of the breast. *The Lancet*, *133*(3421), 571–573.

Parihar, V., Rogers, A., Blain, A. M., Zacharias, S. R. K., Patterson, L. L., & Siyam, M. A.-M. (2022). Reduction in Tamoxifen Metabolites Endoxifen and N-desmethyltamoxifen With Chronic Administration of Low Dose Cannabidiol: A CYP3A4 and CYP2D6 Drug Interaction. *Journal of Pharmacy Practice*, *35*(2), 322–326.

Park, J. H., Jonas, S. F., Bataillon, G., Criscitiello, C., Salgado, R., Loi, S., Viale, G., Lee, H. J., Dieci, M. V, Kim, S.-B., Vincent-Salomon, A., Curigliano, G., André, F., & Michiels, S. (2019). Prognostic value of tumor-infiltrating lymphocytes in patients with early-stage triple-negative breast cancers (TNBC) who did not receive adjuvant chemotherapy. *Annals of Oncology*, *30*(12), 1941–1949.

Park, J. M., Xian, X., Choi, M., Park, H., Cho, Y. K., Lee, I. S., Kim, S. W., & Chung, I. (2011). Antiproliferative mechanism of a cannabinoid agonist by cell cycle arrest in human gastric cancer cells. *Journal of Cellular Biochemistry*, *112*(4), 1192–1205.

Pereira, J. P., An, J., Xu, Y., Huang, Y., & Cyster, J. G. (2009). Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids. *Nature Immunology*, *10*(4), 403–411.

Pérez-Gómez, E., Andradas, C., Blasco-Benito, S., Caffarel, M. M., García-Taboada, E., Villa-Morales, M., Moreno, E., Hamann, S., Martín-Villar, E., Flores, J. M., Wenners, A., Alkatout, I., Klapper, W., Röcken, C., Bronsert, P., Stickeler, E., Staebler, A., Bauer, M., Arnold, N., … Sánchez, C. (2015). Role of Cannabinoid Receptor CB2 in HER2 Pro-

oncogenic Signaling in Breast Cancer. *JNCI: Journal of the National Cancer Institute*, *107*(6).

Pérez-Gómez, E., Andradas, C., Flores, J. M., Quintanilla, M., Paramio, J. M., Guzmán, M., & Sánchez, C. (2013). The orphan receptor GPR55 drives skin carcinogenesis and is upregulated in human squamous cell carcinomas. *Oncogene*, *32*(20), 2534–2542.

Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, Ø., Pergamenschikov, A., Williams, C., Zhu, S. X., Lønning, P. E., Børresen-Dale, A.-L., Brown, P. O., & Botstein, D. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–752.

Picardi, P., Ciaglia, E., Proto, M., & Pisanti, S. (2014). Anandamide inhibits breast tumor-induced angiogenesis. *Translational Medicine @ UniSa*, *10*, 8–12.

Piñeiro, R., Maffucci, T., & Falasca, M. (2011). The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene*, *30*(2), 142–152.

Piper, B. J., Tian, M., Saini, P., Higazy, A., Graham, J., Carbe, C. J., & Bordonaro, M. (2024). Immunotherapy and Cannabis: A Harmful Drug Interaction or Reefer Madness? *Cancers*, *16*(7), 1245.

Pisanti, S., Picardi, P., Prota, L., Proto, M. C., Laezza, C., McGuire, P. G., Morbidelli, L., Gazzerro, P., Ziche, M., Das, A., & Bifulco, M. (2011). Genetic and pharmacologic inactivation of cannabinoid CB1 receptor inhibits angiogenesis. *Blood*, *117*(20), 5541–5550.

Plaks, V., Boldajipour, B., Linnemann, J. R., Nguyen, N. H., Kersten, K., Wolf, Y., Casbon, A.-J., Kong, N., van den Bijgaart, R. J. E., Sheppard, D., Melton, A. C., Krummel, M. F., & Werb, Z. (2015). Adaptive Immune Regulation of Mammary Postnatal Organogenesis. *Developmental Cell*, *34*(5), 493–504.

Pope, C., Kim, S.-K., Marzo, A., Williams, K., Jiang, J., Shen, H., & Lefrançois, L. (2001). Organ-Specific Regulation of the CD8 T Cell Response to *Listeria monocytogenes* Infection. *The Journal of Immunology*, *166*(5), 3402–3409.

Portella, G., Laezza, C., Laccetti, P., De Petrocellis, L., Di Marzo, V., & Bifulco, M. (2003). Inhibitory effects of cannabinoid CB 1 receptor stimulation on tumor growth and metastatic spreading: actions on signals involved in angiogenesis and metastasis. *The FASEB Journal*, *17*(12), 1771–1773.

Qamri, Z., Preet, A., Nasser, M. W., Bass, C. E., Leone, G., Barsky, S. H., & Ganju, R. K. (2009). Synthetic cannabinoid receptor agonists inhibit tumor growth and metastasis of breast cancer. *Molecular Cancer Therapeutics*, *8*(11), 3117–3129.

Qian, B.-Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L. R., Kaiser, E. A., Snyder, L. A., & Pollard, J. W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*, *475*(7355), 222–225.

Qian, J., Wang, C., Wang, B., Yang, J., Wang, Y., Luo, F., Xu, J., Zhao, C., Liu, R., & Chu, Y. (2018). The IFN-γ/PD-L1 axis between T cells and tumor microenvironment: hints for glioma anti-PD-1/PD-L1 therapy. *Journal of Neuroinflammation*, *15*(1), 290.

Qiu, C., Yang, L., Wang, B., Cui, L., Li, C., Zhuo, Y., Zhang, L., Zhang, S., Zhang, Q., & Wang, X. (2019). The role of 2-arachidonoylglycerol in the regulation of the tumor-immune microenvironment in murine models of pancreatic cancer. *Biomedicine & Pharmacotherapy*, *115*, 108952.

Radisky, E. S. (2015). Matrix metalloproteinases as drivers and therapeutic targets in breast cancer. *Frontiers in Bioscience*, *20*(7), 4364.

Rainard, P., Gilbert, F. B., & Germon, P. (2022). Immune defenses of the mammary gland epithelium of dairy ruminants. *Frontiers in Immunology*, *13*.

Rajesh, M., Mukhopadhyay, P., Bátkai, S., Haskó, G., Liaudet, L., Huffman, J. W., Csiszar, A., Ungvari, Z., Mackie, K., Chatterjee, S., & Pacher, P. (2007). CB 2 -receptor stimulation attenuates TNF-α-induced human endothelial cell activation, transendothelial migration of monocytes, and monocyte-endothelial adhesion. *American Journal of Physiology-Heart and Circulatory Physiology*, *293*(4), H2210–H2218.

Rakotoarivelo, V., Mayer, T. Z., Simard, M., Flamand, N., & Di Marzo, V. (2024). The Impact of the CB2 Cannabinoid Receptor in Inflammatory Diseases: An Update. *Molecules*, *29*(14), 3381.

Ramer, R., Fischer, S., Haustein, M., Manda, K., & Hinz, B. (2014). Cannabinoids inhibit angiogenic capacities of endothelial cells via release of tissue inhibitor of matrix metalloproteinases-1 from lung cancer cells. *Biochemical Pharmacology*, *91*(2), 202–216.

Ramirez, S. H., Haskó, J., Skuba, A., Fan, S., Dykstra, H., McCormick, R., Reichenbach, N., Krizbai, I., Mahadevan, A., Zhang, M., Tuma, R., Son, Y.-J., & Persidsky, Y. (2012). Activation of Cannabinoid Receptor 2 Attenuates Leukocyte–Endothelial Cell Interactions and Blood–Brain Barrier Dysfunction under Inflammatory Conditions. *The Journal of Neuroscience*, *32*(12), 4004–4016.

Ravi, J., Elbaz, M., Wani, N. A., Nasser, M. W., & Ganju, R. K. (2016). Cannabinoid receptor-2 agonist inhibits macrophage induced EMT in non-small cell lung cancer by downregulation of EGFR pathway. *Molecular Carcinogenesis*, *55*(12), 2063–2076.

Raz, Y., Cohen, N., Shani, O., Bell, R. E., Novitskiy, S. V., Abramovitz, L., Levy, C., Milyavsky, M., Leider-Trejo, L., Moses, H. L., Grisaru, D., & Erez, N. (2018). Bone marrow–derived fibroblasts are a functionally distinct stromal cell population in breast cancer. *Journal of Experimental Medicine*, *215*(12), 3075–3093.

Reich, N., & Hölscher, C. (2024). Cholecystokinin (CCK): a neuromodulator with therapeutic potential in Alzheimer's and Parkinson's disease. *Frontiers in Neuroendocrinology*, *73*, 101122.

Rezende, B., Alencar, A. K. N., de Bem, G. F., Fontes-Dantas, F. L., & Montes, G. C. (2023). Endocannabinoid System: Chemical Characteristics and Biological Activity. *Pharmaceuticals*, *16*(2), 148.

Riggio, A. I., Varley, K. E., & Welm, A. L. (2021). The lingering mysteries of metastatic recurrence in breast cancer. *British Journal of Cancer*, *124*(1), 13–26.

Robinson, R. H., Meissler, J. J., Breslow-Deckman, J. M., Gaughan, J., Adler, M. W., & Eisenstein, T. K. (2013). Cannabinoids Inhibit T-cells via Cannabinoid Receptor 2 in an In Vitro Assay for Graft Rejection, the Mixed Lymphocyte Reaction. *Journal of Neuroimmune Pharmacology*, *8*(5), 1239–1250.

Rockwell, C. E., Raman, P., Kaplan, B. L. F., & Kaminski, N. E. (2008). A COX-2 metabolite of the endogenous cannabinoid, 2-arachidonyl glycerol, mediates suppression of IL-2 secretion in activated Jurkat T cells. *Biochemical Pharmacology*, *76*(3), 353–361.

Rockwell, C. E., Snider, N. T., Thompson, J. T., Vanden Heuvel, J. P., & Kaminski, N. E. (2006). Interleukin-2 Suppression by 2-Arachidonyl Glycerol Is Mediated through Peroxisome Proliferator-Activated Receptor γ Independently of Cannabinoid Receptors 1 and 2. *Molecular Pharmacology*, *70*(1), 101–111.

Rodríguez-Bejarano, O. H., Parra-López, C., & Patarroyo, M. A. (2024). A review concerning the breast cancer-related tumour microenvironment. *Critical Reviews in Oncology/Hematology*, *199*, 104389.

Ruhl, T., Lippold, E. F., Christer, T., Schaefer, B., Kim, B.-S., & Beier, J. P. (2021). Genetic deletion of the cannabinoid receptors CB1 and CB2 enhances inflammation with diverging effects on skin wound healing in mice. *Life Sciences*, *285*, 120018.

Salazar, M., Carracedo, A., Salanueva, Í. J., Hernández-Tiedra, S., Lorente, M., Egia, A., Vázquez, P., Blázquez, C., Torres, S., García, S., Nowak, J., Fimia, G. M., Piacentini, M., Cecconi, F., Pandolfi, P. P., González-Feria, L., Iovanna, J. L., Guzmán, M., Boya, P., & Velasco, G. (2009). Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. *Journal of Clinical Investigation*, *119*(5), 1359–1372.

Salgado, R., Denkert, C., Demaria, S., Sirtaine, N., Klauschen, F., Pruneri, G., Wienert, S., Van den Eynden, G., Baehner, F. L., Penault-Llorca, F., Perez, E. A., Thompson, E. A., Symmans, W. F., Richardson, A. L., Brock, J., Criscitiello, C., Bailey, H., Ignatiadis, M., Floris, G., … Loi, S. (2015). The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Annals of Oncology*, *26*(2), 259–271.

Sánchez, C., de Ceballos, M. L., Gomez del Pulgar, T., Rueda, D., Corbacho, C., Velasco, G., Galve-Roperh, I., Huffman, J. W., Ramón y Cajal, S., & Guzmán, M. (2001). Inhibition of glioma growth in vivo by selective activation of the CB(2) cannabinoid receptor. *Cancer Research*, *61*(15), 5784–5789.

Sánchez, C., Galve-Roperh, I., Canova, C., Brachet, P., & Guzmán, M. (1998). Δ 9 -Tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Letters*, *436*(1), 6–10.

Sánchez, M. G., Sánchez, A. M., Ruiz-Llorente, L., & Díaz-Laviada, I. (2003). Enhancement of androgen receptor expression induced by (*R*)-methanandamide in prostate LNCaP cells. *FEBS Letters*, *555*(3), 561–566.

Sarsembayeva, A., Kienzl, M., Gruden, E., Ristic, D., Maitz, K., Valadez-Cosmes, P., Santiso, A., Hasenoehrl, C., Brcic, L., Lindenmann, J., Kargl, J., & Schicho, R. (2023).

Cannabinoid receptor 2 plays a pro-tumorigenic role in non-small cell lung cancer by limiting anti-tumor activity of CD8+ T and NK cells. *Frontiers in Immunology*, *13*.

Scarlett, K. A., White, E.-S. Z., Coke, C. J., Carter, J. R., Bryant, L. K., & Hinton, C. V. (2018). Agonist-induced CXCR4 and CB2 Heterodimerization Inhibits Gα13/RhoA-mediated Migration. *Molecular Cancer Research*, *16*(4), 728–739.

Sceneay, J., Chow, M. T., Chen, A., Halse, H. M., Wong, C. S. F., Andrews, D. M., Sloan, E. K., Parker, B. S., Bowtell, D. D., Smyth, M. J., & Möller, A. (2012). Primary Tumor Hypoxia Recruits CD11b+/Ly6Cmed/Ly6G+ Immune Suppressor Cells and Compromises NK Cell Cytotoxicity in the Premetastatic Niche. *Cancer Research*, *72*(16), 3906–3911.

Schatz, A. R., Lee, M., Condie, R. B., Pulaski, J. T., & Kaminski, N. E. (1997). Cannabinoid Receptors CB1 and CB2: A Characterization of Expression and Adenylate Cyclase Modulation within the Immune System. *Toxicology and Applied Pharmacology*, *142*(2), 278–287.

Schley, M., Ständer, S., Kerner, J., Vajkoczy, P., Schüpfer, G., Dusch, M., Schmelz, M., & Konrad, C. (2009). Predominant CB2 receptor expression in endothelial cells of glioblastoma in humans. *Brain Research Bulletin*, *79*(5), 333–337.

Schloss, J., Lacey, J., Sinclair, J., Steel, A., Sughrue, M., Sibbritt, D., & Teo, C. (2021). A Phase 2 Randomised Clinical Trial Assessing the Tolerability of Two Different Ratios of Medicinal Cannabis in Patients With High Grade Gliomas. *Frontiers in Oncology*, *11*.

Schmid, M. C., Khan, S. Q., Kaneda, M. M., Pathria, P., Shepard, R., Louis, T. L., Anand, S., Woo, G., Leem, C., Faridi, M. H., Geraghty, T., Rajagopalan, A., Gupta, S., Ahmed, M., Vazquez-Padron, R. I., Cheresh, D. A., Gupta, V., & Varner, J. A. (2018). Integrin CD11b activation drives anti-tumor innate immunity. *Nature Communications*, *9*(1), 5379.

Schmid, P., Adams, S., Rugo, H. S., Schneeweiss, A., Barrios, C. H., Iwata, H., Diéras, V., Hegg, R., Im, S.-A., Shaw Wright, G., Henschel, V., Molinero, L., Chui, S. Y., Funke, R., Husain, A., Winer, E. P., Loi, S., & Emens, L. A. (2018). Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. *New England Journal of Medicine*, *379*(22), 2108–2121.

Schmid, P. C., Wold, L. E., Krebsbach, R. J., Berdyshev, E. V., & Schmid, H. H. O. (2002). Anandamide and other *N* -acylethanolamines in human tumors. *Lipids*, *37*(9), 907–912.

Schmid, P., Cortes, J., Pusztai, L., McArthur, H., Kümmel, S., Bergh, J., Denkert, C., Park, Y. H., Hui, R., Harbeck, N., Takahashi, M., Foukakis, T., Fasching, P. A., Cardoso, F., Untch, M., Jia, L., Karantza, V., Zhao, J., Aktan, G., … O'Shaughnessy, J. (2020). Pembrolizumab for Early Triple-Negative Breast Cancer. *New England Journal of Medicine*, *382*(9), 810–821.

Schmiedel, B. J., Singh, D., Madrigal, A., Valdovino-Gonzalez, A. G., White, B. M., Zapardiel-Gonzalo, J., Ha, B., Altay, G., Greenbaum, J. A., McVicker, G., Seumois, G., Rao, A., Kronenberg, M., Peters, B., & Vijayanand, P. (2018). Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell*, *175*(6), 1701-1715.e16.

Schurman, L. D., Lu, D., Kendall, D. A., Howlett, A. C., & Lichtman, A. H. (2019). *Molecular Mechanism and Cannabinoid Pharmacology* (pp. 323–353).

Scott, K. A., Dalgleish, A. G., & Liu, W. M. (2014). The Combination of Cannabidiol and Δ9-Tetrahydrocannabinol Enhances the Anticancer Effects of Radiation in an Orthotopic Murine Glioma Model. *Molecular Cancer Therapeutics*, *13*(12), 2955–2967.

Scott, K. A., Dalgleish, A. G., & Liu, W. M. (2017). Anticancer effects of phytocannabinoids used with chemotherapy in leukaemia cells can be improved by altering the sequence of their administration. *International Journal of Oncology*, *51*(1), 369–377.

Sexton, M., Garcia, J. M., Jatoi, A., Clark, C. S., & Wallace, M. S. (2021). The Management of Cancer Symptoms and Treatment-Induced Side Effects With Cannabis or Cannabinoids. *JNCI Monographs*, *2021*(58), 86–98.

Shao, Z., Yin, J., Chapman, K., Grzemska, M., Clark, L., Wang, J., & Rosenbaum, D. M. (2016). High-resolution crystal structure of the human CB1 cannabinoid receptor. *Nature*, *540*(7634), 602–606.

Shen, M., Wang, J., Yu, W., Zhang, C., Liu, M., Wang, K., Yang, L., Wei, F., Wang, S. E., Sun, Q., & Ren, X. (2018). A novel MDSC-induced PD-1 − PD-L1 + B-cell subset in breast tumor microenvironment possesses immuno-suppressive properties. *OncoImmunology*, *7*(4).

Shi, X., Young, C. D., Zhou, H., & Wang, X.-J. (2020). Transforming Growth Factor-β Signaling in Fibrotic Diseases and Cancer-Associated Fibroblasts. *Biomolecules*, *10*(12), 1666.

Shiratsuchi, A., Watanabe, I., Yoshida, H., & Nakanishi, Y. (2008). Involvement of cannabinoid receptor CB2 in dectin-1-mediated macrophage phagocytosis. *Immunology & Cell Biology*, *86*(2), 179–184.

Shrivastava, A., Kuzontkoski, P. M., Groopman, J. E., & Prasad, A. (2011). Cannabidiol Induces Programmed Cell Death in Breast Cancer Cells by Coordinating the Cross-talk between Apoptosis and Autophagy. *Molecular Cancer Therapeutics*, *10*(7), 1161–1172.

Simard, M., Archambault, A.-S., Lavoie, J.-P. C., Dumais, É., Di Marzo, V., & Flamand, N. (2022). Biosynthesis and metabolism of endocannabinoids and their congeners from the monoacylglycerol and N-acyl-ethanolamine families. *Biochemical Pharmacology*, *205*, 115261.

Simsek, T., Bal Albayrak, M., Akpinar, G., Canturk, N., & Kasap, M. (2023). Downregulated GPD1 and MAGL protein levels as potential biomarkers for the metastasis of triple-negative breast tumors to axillary lymph nodes. *Oncology Letters*, *27*(1), 34.

Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., de Petrocellis, L., Ullmer, C., Rothenhäusler, B., Perret, C., van Gils, N., Finlay, D., MacDonald, C., Chicca, A., Gens, M. D., Stuart, J., de Vries, H., Mastrangelo, N., Xia, L., Alachouzos, G., … van der Stelt, M. (2017). Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. *Nature Communications*, *8*(1), 13958.

Song, C., & Howlett, A. C. (1995). Rat brain cannabinoid receptors are N-linked glycosylated proteins. *Life Sciences*, *56*(23–24), 1983–1989.

Song, Q., Zhang, W., Shi, D., Zhang, Z., Zhao, Q., Wang, M., Huang, M., Meng, J., Cui, W., & Luo, X. (2023). Overexpression of cannabinoid receptor 2 is associated with human breast cancer proliferation, apoptosis, chemosensitivity and prognosis via the PI3K/Akt/mTOR signaling pathway. *Cancer Medicine*, *12*(12), 13538–13550.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lønning, P. E., & Børresen-Dale, A.-L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences*, *98*(19), 10869–10874.

Soroceanu, L., Murase, R., Limbad, C., Singer, E., Allison, J., Adrados, I., Kawamura, R., Pakdel, A., Fukuyo, Y., Nguyen, D., Khan, S., Arauz, R., Yount, G. L., Moore, D. H., Desprez, P.-Y., & McAllister, S. D. (2013). Id-1 Is a Key Transcriptional Regulator of Glioblastoma Aggressiveness and a Novel Therapeutic Target. *Cancer Research*, *73*(5), 1559–1569.

Specter, S. C., Klein, T. W., Newton, C., Mondragon, M., Widen, R., & Friedman, H. (1986). Marijuana effects on immunity: Suppression of human natural killer cell activity by delta-9-tetrahydrocannabinol. *International Journal of Immunopharmacology*, *8*(7), 741–745.

Springs, A. E. B., Karmaus, P. W. F., Crawford, R. B., Kaplan, B. L. F., & Kaminski, N. E. (2008). Effects of targeted deletion of cannabinoid receptors CB1 and CB2 on immune competence and sensitivity to immune modulation by Δ9-tetrahydrocannabinol. *Journal of Leukocyte Biology*, *84*(6), 1574–1584.

St. Paul, M., & Ohashi, P. S. (2020). The Roles of CD8+ T Cell Subsets in Antitumor Immunity. *Trends in Cell Biology*, *30*(9), 695–704.

Stanton, S. E., & Disis, M. L. (2016). Clinical significance of tumor-infiltrating lymphocytes in breast cancer. *Journal for ImmunoTherapy of Cancer*, *4*(1), 59.

Stefano, G. B., Bilfinger, T. V, Rialas, C. M., & Deutsch, D. G. (2000). 2-arachidonyl-glycerol stimulates nitric oxide release from human immune and vascular tissues and invertebrate immunocytes by cannabinoid receptor 1. *Pharmacological Research*, *42*(4), 317–322.

Stenström, J., Hedenfalk, I., & Hagerling, C. (2021). Regulatory T lymphocyte infiltration in metastatic breast cancer—an independent prognostic factor that changes with tumor progression. *Breast Cancer Research*, *23*(1), 27.

Stojanovic, B., Gajovic, N., Jurisevic, M., Stojanovic, M. D., Jovanovic, M., Jovanovic, I., Stojanovic, B. S., & Milosevic, B. (2023). Decoding the IL-33/ST2 Axis: Its Impact on the Immune Landscape of Breast Cancer. *International Journal of Molecular Sciences*, *24*(18), 14026.

Su, S., Liu, Q., Chen, J., Chen, J., Chen, F., He, C., Huang, D., Wu, W., Lin, L., Huang, W., Zhang, J., Cui, X., Zheng, F., Li, H., Yao, H., Su, F., & Song, E. (2014). A Positive

Feedback Loop between Mesenchymal-like Cancer Cells and Macrophages Is Essential to Breast Cancer Metastasis. *Cancer Cell*, *25*(5), 605–620.

Suk, K.-T., Mederacke, I., Gwak, G.-Y., Cho, S. W., Adeyemi, A., Friedman, R., & Schwabe, R. F. (2016). Opposite roles of cannabinoid receptors 1 and 2 in hepatocarcinogenesis. *Gut*, *65*(10), 1721–1732.

Sun, Y.-P., Ke, Y.-L., & Li, X. (2022). Prognostic value of CD8 + tumor-infiltrating T cells in patients with breast cancer: A systematic review and meta-analysis. *Oncology Letters*, *25*(1), 39.

Szekely, B., Bossuyt, V., Li, X., Wali, V. B., Patwardhan, G. A., Frederick, C., Silber, A., Park, T., Harigopal, M., Pelekanou, V., Zhang, M., Yan, Q., Rimm, D. L., Bianchini, G., Hatzis, C., & Pusztai, L. (2018). Immunological differences between primary and metastatic breast cancer. *Annals of Oncology*, *29*(11), 2232–2239.

Taftaf, R., Liu, X., Singh, S., Jia, Y., Dashzeveg, N. K., Hoffmann, A. D., El-Shennawy, L., Ramos, E. K., Adorno-Cruz, V., Schuster, E. J., Scholten, D., Patel, D., Zhang, Y., Davis, A. A., Reduzzi, C., Cao, Y., D'Amico, P., Shen, Y., Cristofanilli, M., … Liu, H. (2021). ICAM1 initiates CTC cluster formation and trans-endothelial migration in lung metastasis of breast cancer. *Nature Communications*, *12*(1), 4867.

Taha, T., Meiri, D., Talhamy, S., Wollner, M., Peer, A., & Bar-Sela, G. (2019). Cannabis Impacts Tumor Response Rate to Nivolumab in Patients with Advanced Malignancies. *The Oncologist*, *24*(4), 549–554.

Tau, G., & Rothman, P. (1999). Biologic functions of the IFN-γ receptors. *Allergy*, *54*(12), 1233–1251.

Taylor, B. C., & Balko, J. M. (2022). Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response. *Frontiers in Immunology*, *13*.

Teng, M. W. L., Westwood, J. A., Darcy, P. K., Sharkey, J., Tsuji, M., Franck, R. W., Porcelli, S. A., Besra, G. S., Takeda, K., Yagita, H., Kershaw, M. H., & Smyth, M. J. (2007). Combined Natural Killer T-Cell–Based Immunotherapy Eradicates Established Tumors in Mice. *Cancer Research*, *67*(15), 7495–7504.

Thacker, G., Henry, S., Nandi, A., Debnath, R., Singh, S., Nayak, A., Susnik, B., Boone, M. M., Zhang, Q., Kesmodel, S. B., Gumber, S., Das, G. M., Kambayashi, T., Dos Santos, C. O., & Chakrabarti, R. (2023). Immature natural killer cells promote progression of triple-negative breast cancer. *Science Translational Medicine*, *15*(686).

Theocharis, S., Giaginis, C., Alexandrou, P., Rodriguez, J., Tasoulas, J., Danas, E., Patsouris, E., & Klijanienko, J. (2016). Evaluation of cannabinoid CB1 and CB2 receptors expression in mobile tongue squamous cell carcinoma: associations with clinicopathological parameters and patients' survival. *Tumor Biology*, *37*(3), 3647–3656.

Thomas, R., Al-Khadairi, G., Roelands, J., Hendrickx, W., Dermime, S., Bedognetti, D., & Decock, J. (2018). NY-ESO-1 Based Immunotherapy of Cancer: Current Perspectives. *Frontiers in Immunology*, *9*.

Todd, A. R. (1946). Hashish. *Experientia*, *2*(2), 55–60.

Torres, S., Lorente, M., Rodríguez-Fornés, F., Hernández-Tiedra, S., Salazar, M., García-Taboada, E., Barcia, J., Guzmán, M., & Velasco, G. (2011). A Combined Preclinical Therapy of Cannabinoids and Temozolomide against Glioma. *Molecular Cancer Therapeutics*, *10*(1), 90–103.

Treilleux, I., Blay, J.-Y., Bendriss-Vermare, N., Ray-Coquard, I., Bachelot, T., Guastalla, J.-P., Bremond, A., Goddard, S., Pin, J.-J., Barthelemy-Dubois, C., & Lebecque, S. (2004). Dendritic Cell Infiltration and Prognosis of Early Stage Breast Cancer. *Clinical Cancer Research*, *10*(22), 7466–7474.

Triulzi, T., De Cecco, L., Sandri, M., Prat, A., Giussani, M., Paolini, B., Carcangiu, M. L., Canevari, S., Bottini, A., Balsari, A., Menard, S., Generali, D., Campiglio, M., Di Cosimo, S., & Tagliabue, E. (2015). Whole-transcriptome analysis links trastuzumab sensitivity of breast tumors to both HER2 dependence and immune cell infiltration. *Oncotarget*, *6*(29), 28173–28182.

Tsang, J. Y. S., & Tse, G. M. (2020). Molecular Classification of Breast Cancer. *Advances in Anatomic Pathology*, *27*(1), 27–35.

Tundidor, I., Seijo-Vila, M., Blasco-Benito, S., Rubert-Hernández, M., Adámez, S., Andradas, C., Manzano, S., Álvarez-López, I., Sarasqueta, C., Villa-Morales, M., González-Lois, C., Ramírez-Medina, E., Almoguera, B., Sánchez-López, A. J., Bindila, L., Hamann, S., Arnold, N., Röcken, C., Heras-Murillo, I., … Pérez-Gómez, E. (2023). Identification of fatty acid amide hydrolase as a metastasis suppressor in breast cancer. *Nature Communications*, *14*(1), 3130.

Turcotte, C., Blanchet, M.-R., Laviolette, M., & Flamand, N. (2016). The CB2 receptor and its role as a regulator of inflammation. *Cellular and Molecular Life Sciences*, *73*(23), 4449–4470.

Twelves, C., Sabel, M., Checketts, D., Miller, S., Tayo, B., Jove, M., Brazil, L., & Short, S. C. (2021). A phase 1b randomised, placebo-controlled trial of nabiximols cannabinoid oromucosal spray with temozolomide in patients with recurrent glioblastoma. *British Journal of Cancer*, *124*(8), 1379–1387.

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A.-K., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., … Pontén, F. (2015). Tissue-based map of the human proteome. *Science*, *347*(6220).

Vara, D., Salazar, M., Olea-Herrero, N., Guzmán, M., Velasco, G., & Díaz-Laviada, I. (2011). Erratum: Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy. *Cell Death & Differentiation*, *18*(7), 1237–1237.

Velasco, G., Sánchez, C., & Guzmán, M. (2016). Anticancer Mechanisms of Cannabinoids. *Current Oncology*, *23*(11), 23–32.

Waissengrin, B., Leshem, Y., Taya, M., Meiri, D., Merimsky, O., Shamai, S., Wolf, I., & Rubinek, T. (2023). The use of medical cannabis concomitantly with immune

checkpoint inhibitors in non-small cell lung cancer: A sigh of relief? *European Journal of Cancer*, *180*, 52–61.

Wang, B., Liu, J., Han, Y., Deng, Y., Li, J., & Jiang, Y. (2022). The Presence of Tertiary Lymphoid Structures Provides New Insight Into the Clinicopathological Features and Prognosis of Patients With Breast Cancer. *Frontiers in Immunology*, *13*.

Wang, N., Liu, W., Zheng, Y., Wang, S., Yang, B., Li, M., Song, J., Zhang, F., Zhang, X., Wang, Q., & Wang, Z. (2018). CXCL1 derived from tumor-associated macrophages promotes breast cancer metastasis via activating NF-κB/SOX4 signaling. *Cell Death & Disease*, *9*(9), 880.

Wang, X., Collet, L., Rediti, M., Debien, V., De Caluwé, A., Venet, D., Romano, E., Rothé, F., Sotiriou, C., & Buisseret, L. (2023). Predictive Biomarkers for Response to Immunotherapy in Triple Negative Breast Cancer: Promises and Challenges. *Journal of Clinical Medicine*, *12*(3), 953.

Wen, Z., Liu, T., Xu, X., Acharya, N., Shen, Z., Lu, Y., Xu, J., Guo, K., Shen, S., Zhao, Y., Wang, P., Li, S., Chen, W., Li, H., Ding, Y., Shang, M., Guo, H., Hou, Y., Cui, B., … Xiao, P. (2025). Interleukin-16 enhances anti-tumor immune responses by establishing a Th1 cell-macrophage crosstalk through reprogramming glutamine metabolism in mice. *Nature Communications*, *16*(1), 2362.

WHO. (2019). *WHO Classification of Tumours of the Breast, 5th Edition*.

Whynot, E. G., Tomko, A. M., & Dupré, D. J. (2023). Anticancer properties of cannabidiol and Δ9-tetrahydrocannabinol and synergistic effects with gemcitabine and cisplatin in bladder cancer cell lines. *Journal of Cannabis Research*, *5*(1), 7.

Wiede, F., Lu, K.-H., Du, X., Zeissig, M. N., Xu, R., Goh, P. K., Xirouchaki, C. E., Hogarth, S. J., Greatorex, S., Sek, K., Daly, R. J., Beavis, P. A., Darcy, P. K., Tonks, N. K., & Tiganis, T. (2022). PTP1B Is an Intracellular Checkpoint that Limits T-cell and CAR T-cell Antitumor Immunity. *Cancer Discovery*, *12*(3), 752–773.

Williams, S. A., Harata-Lee, Y., Comerford, I., Anderson, R. L., Smyth, M. J., & McColl, S. R. (2010). Multiple functions of CXCL12 in a syngeneic model of breast cancer. *Molecular Cancer*, *9*(1), 250.

Winkler, J., Abisoye-Ogunniyan, A., Metcalf, K. J., & Werb, Z. (2020). Concepts of extracellular matrix remodelling in tumour progression and metastasis. *Nature Communications*, *11*(1), 5120.

Winslow, S., Leandersson, K., Edsjö, A., & Larsson, C. (2015). Prognostic stromal gene signatures in breast cancer. *Breast Cancer Research*, *17*(1), 23.

Wortman, J. C., He, T.-F., Solomon, S., Zhang, R. Z., Rosario, A., Wang, R., Tu, T. Y., Schmolze, D., Yuan, Y., Yost, S. E., Li, X., Levine, H., Atwal, G., Lee, P. P., & Yu, C. C. (2021). Spatial distribution of B cells and lymphocyte clusters as a predictor of triple-negative breast cancer outcome. *Npj Breast Cancer*, *7*(1), 84.

Wu, L., Saxena, S., Goel, P., Prajapati, D. R., Wang, C., & Singh, R. K. (2020). Breast Cancer Cell–Neutrophil Interactions Enhance Neutrophil Survival and Pro-Tumorigenic Activities. *Cancers*, *12*(10), 2884.

Xiang, W., Shi, R., Kang, X., Zhang, X., Chen, P., Zhang, L., Hou, A., Wang, R., Zhao, Y., Zhao, K., Liu, Y., Ma, Y., Luo, H., Shang, S., Zhang, J., He, F., Yu, S., Gan, L., Shi, C., … Miao, H. (2018). Monoacylglycerol lipase regulates cannabinoid receptor 2-dependent macrophage activation and cancer progression. *Nature Communications*, *9*(1), 2574.

Xiang, X., Poliakov, A., Liu, C., Liu, Y., Deng, Z., Wang, J., Cheng, Z., Shah, S. V., Wang, G., Zhang, L., Grizzle, W. E., Mobley, J., & Zhang, H. (2009). Induction of myeloid-derived suppressor cells by tumor exosomes. *International Journal of Cancer*, *124*(11), 2621–2633.

Xing, C., Zhuang, Y., Xu, T.-H., Feng, Z., Zhou, X. E., Chen, M., Wang, L., Meng, X., Xue, Y., Wang, J., Liu, H., McGuire, T. F., Zhao, G., Melcher, K., Zhang, C., Xu, H. E., & Xie, X.-Q. (2020). Cryo-EM Structure of the Human Cannabinoid Receptor CB2-Gi Signaling Complex. *Cell*, *180*(4), 645-654.e13.

Xiong, X., Chen, S., Shen, J., You, H., Yang, H., Yan, C., Fang, Z., Zhang, J., Cai, X., Dong, X., Kang, T., Li, W., & Zhou, P. (2022). Cannabis suppresses antitumor immunity by inhibiting JAK/STAT signaling in T cells through CNR2. *Signal Transduction and Targeted Therapy*, *7*(1), 99.

Xiong, Y., & Bosselut, R. (2012). CD4–CD8 differentiation in the thymus: connecting circuits and building memories. *Current Opinion in Immunology*, *24*(2), 139–145.

Xu, D., Wang, J., Zhou, Z., He, Z., & Zhao, Q. (2015). Cannabinoid WIN55, 212-2 induces cell cycle arrest and inhibits the proliferation and migration of human BEL7402 hepatocellular carcinoma cells. *Molecular Medicine Reports*, *12*(6), 7963–7970.

Xu, H., & Xu, B. (2023). Breast cancer: Epidemiology, risk factors and screening. *Chinese Journal of Cancer Research*, *35*(6), 565–583.

Xu, X., Liu, Y., Huang, S., Liu, G., Xie, C., Zhou, J., Fan, W., Li, Q., Wang, Q., Zhong, D., & Miao, X. (2006). Overexpression of cannabinoid receptors CB1 and CB2 correlates with improved prognosis of patients with hepatocellular carcinoma. *Cancer Genetics and Cytogenetics*, *171*(1), 31–38.

Xuan, Q., Wang, J., Nanding, A., Wang, Z., Liu, H., Lian, X., & Zhang, Q. (2014). Tumor-Associated Macrophages are Correlated with Tamoxifen Resistance in the Postmenopausal Breast Cancer Patients. *Pathology & Oncology Research*, *20*(3), 619–624.

Yan, M., Jene, N., Byrne, D., Millar, E. K., O'Toole, S. A., McNeil, C. M., Bates, G. J., Harris, A. L., Banham, A. H., Sutherland, R. L., & Fox, S. B. (2011). Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers. *Breast Cancer Research*, *13*(2), R47.

Yang, J., Hills, D., Taylor, E., Pfeffer, K., Ure, J., & Medvinsky, A. (2008). Transgenic tools for analysis of the haematopoietic system: Knock-in CD45 reporter and deletor mice. *Journal of Immunological Methods*, *337*(2), 81–87.

Yi, J. I., Schneider, J., Lim, S. T., Park, B., & Suh, Y. J. (2024). Interferon-Gamma Secretion Is Significantly Decreased in Stage III Breast Cancer Patients. *International Journal of Molecular Sciences*, *25*(8), 4561.

Yoshida, H., Lareau, C. A., Ramirez, R. N., Rose, S. A., Maier, B., Wroblewska, A., Desland, F., Chudnovskiy, A., Mortha, A., Dominguez, C., Tellier, J., Kim, E., Dwyer, D., Shinton, S., Nabekura, T., Qi, Y., Yu, B., Robinette, M., Kim, K.-W., … Benoist, C. (2019). The cis-Regulatory Atlas of the Mouse Immune System. *Cell*, *176*(4), 897-912.e20.

Yoshimura, T., Li, C., Wang, Y., & Matsukawa, A. (2023). The chemokine monocyte chemoattractant protein-1/CCL2 is a promoter of breast cancer metastasis. *Cellular & Molecular Immunology*, *20*(7), 714–738.

Young, A. P., Szczesniak, A.-M., Hsu, K., Kelly, M. E. M., & Denovan-Wright, E. M. (2024). Enantiomeric Agonists of the Type 2 Cannabinoid Receptor Reduce Retinal Damage during Proliferative Vitreoretinopathy and Inhibit Hyperactive Microglia *In Vitro*. *ACS Pharmacology & Translational Science*, *7*(5), 1348–1363.

Yuan, C. Y., Zhou, V., Sauber, G., Stollenwerk, T., Komorowski, R., López, A., Tolón, R. M., Romero, J., Hillard, C. J., & Drobyski, W. R. (2021). Signaling through the type 2 cannabinoid receptor regulates the severity of acute and chronic graft-versus-host disease. *Blood*, *137*(9), 1241–1255.

Yuan, M., Kiertscher, S. M., Cheng, Q., Zoumalan, R., Tashkin, D. P., & Roth, M. D. (2002). Δ9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. *Journal of Neuroimmunology*, *133*(1–2), 124–131.

Yuan, Z.-Y., Luo, R.-Z., Peng, R.-J., Wang, S.-S., & Xue, C. (2014). High infiltration of tumor-associated macrophages in triple-negative breast cancer is associated with a higher risk of distant metastasis. *OncoTargets and Therapy*, 1475.

Zagami, P., & Carey, L. A. (2022). Triple negative breast cancer: Pitfalls and progress. *Npj Breast Cancer*, *8*(1), 95.

Zareinejad, M., Mehdipour, F., Roshan-Zamir, M., Faghih, Z., & Ghaderi, A. (2023). Dual Functions of T Lymphocytes in Breast Carcinoma: From Immune Protection to Orchestrating Tumor Progression and Metastasis. *Cancers*, *15*(19), 4771.

Zhang, C., Wu, F., Lu, X., Wang, S., Wu, M., Chen, N., Fan, S., & Wei, W. (2024). Peripheral NK cell count predicts response and prognosis in breast cancer patients underwent neoadjuvant chemotherapy. *Frontiers in Immunology*, *15*.

Zhang, H., Yu, Y., Zhou, L., Ma, J., Tang, K., Xu, P., Ji, T., Liang, X., Lv, J., Dong, W., Zhang, T., Chen, D., Xie, J., Liu, Y., & Huang, B. (2018). Circulating Tumor Microparticles Promote Lung Metastasis by Reprogramming Inflammatory and Mechanical Niches via a Macrophage-Dependent Pathway. *Cancer Immunology Research*, *6*(9), 1046–1056.

Zhang, L., Wang, X. I., Ding, J., Sun, Q., & Zhang, S. (2019). The predictive and prognostic value of Foxp3+/CD25+ regulatory T cells and PD-L1 expression in triple negative breast cancer. *Annals of Diagnostic Pathology*, *40*, 143–151.

Zhang, W., Zhong, W., Wang, B., Yang, J., Yang, J., Yu, Z., Qin, Z., Shi, A., Xu, W., Zheng, C., Schuchter, L. M., Karakousis, G. C., Mitchell, T. C., Amaravadi, R., Herlyn, M., Dong, H., Gimotty, P. A., Daaboul, G., Xu, X., & Guo, W. (2022). ICAM-1-mediated adhesion is a prerequisite for exosome-induced T cell suppression. *Developmental Cell*, *57*(3), 329-343.e7.

Zheng, Z. M., Specter, S., & Friedman, H. (1992). Inhibition by delta-9-tetrahydrocannabinol of tumor necrosis factor alpha production by mouse and human macrophages. *International Journal of Immunopharmacology*, *14*(8), 1445–1452.

Zhou, L., Zhou, S., Yang, P., Tian, Y., Feng, Z., Xie, X.-Q., & Liu, Y. (2018). Targeted inhibition of the type 2 cannabinoid receptor is a novel approach to reduce renal fibrosis. *Kidney International*, *94*(4), 756–772.

Zhou, Q., Xu, J., Xu, Y., Sun, S., & Chen, J. (2023). Role of ICAM1 in tumor immunity and prognosis of triple-negative breast cancer. *Frontiers in Immunology*, *14*.

Zhu, L. X., Sharma, S., Stolina, M., Gardner, B., Roth, M. D., Tashkin, D. P., & Dubinett, S. M. (2000). Δ-9-Tetrahydrocannabinol Inhibits Antitumor Immunity by a CB2 Receptor-Mediated, Cytokine-Dependent Pathway. *The Journal of Immunology*, *165*(1), 373–380.

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 and Tissue Research*, *347*(1), 73–84.