UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Exploring association of melanoma-specific Bcl-xL with tumor immune microenvironment

Estudio del impacto de la expresión de Bcl-xL en melanoma sobre el microambiente inmunitario tumoral

D.ª Anna Maria Lucianò

2023

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UNIVERSIDAD DE MURCIA

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Aprobado por la Comisión General de Doctorado el 19-10-2022

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Exploring association of melanoma-specific Bcl-xL with tumor immune microenvironment Estudio del impacto de la expresión de Bcl-xL en melanoma sobre el microambiente inmunitario tumoral

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ABBREVIATIONS

A1/Bfl-1 Bcl-2-Related Protein A1/Bcl-2-Related Isolated from Fetal Liver-11

AP1 Activator Protein 1

APAF1: Apoptotic Protease Activating Factor-1

ARF: ADP Ribosylation Factor

ARG: Arginase 1

BAD: Bcl-2 Associated Agonist of Cell Death

BAK: Bcl-2 Antagonist/Killer

BAX: Bcl-2-Associated X Protein

Bcl-2: B-Cell Lymphoma 2

Bcl-w: B-Cell Lymphoma-W

Bcl-xL: B-Cell Lymphoma-Extralarge

BH: Bcl-2 Homology Domains

BIM: Bcl-2-Interacting Mediator Of Cell Death

BMM: Bone Marrow Macrophages

BRAF: V-Raf Murine Sarcoma Viral Oncogene

C: Cytosine

CAFs: Cancer Associated Fibroblasts

CCL: C-C Motif Chemokine Ligand

CDKN2: Cyclin Dependent Kinase Inhibitor 2A

CDX: Cell Line-Derived Xenograft Model

CM: Culture Medium

CSF: Colony Stimulating Factor

CTCs: Circulating Tumor Cells

CXCL: C-X-C Motif Chemokine Ligand

CXCR: C-X-C Motif Chemokine Receptor

DCs: Dendritic Cells

DNA: Deoxyribonucleic Acid

DPF: Days Post Fertilization

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetra-Acetic Acid

EGF: Epidermal Growth Factor

EMT: Epithelial to Mesenchymal Transition

ERK: Extracellular Signal-Regulated Kinase

ETS: Erythroblast Transformation Specific

FBS: Fetal Bovin Serum

FDA: Food and Drug Administration

FGF: Fibroblast Growth Factors

GATA: Globin Transcription Factor

GC: Gastric Cancer

MSCs: Mesenchymal Stem Cells

GEMs: Genetically Engineered Models

GFP: Green Fluorescent Protein

HGH: Hepatocyte Growth Factor

HIF: Hypoxia Inducible Factor

HLA: Human Leukocyte Antigen

HnRNP: Heterogeneous Nuclear Ribonucleoproteins

HPF: House Post-Fertilization

IGF: Insulin Growth Factor

IHC: Immunohistochemical

IKB: Inhibitor off NF-κB

IL: Interleukin

INF: Interferon

iNOS: inducible Nitric Oxide Synthase

JAK: Janus Kinase

JNK: Jun Amino-Terminal Kinases

LSM: Like Spliceosomal

MAPK: Mitogen-Activated Protein Kinase

MCL: Myeloid Cell Leukemia

MCP: Monocyte Chemoattractant Protein

M-CSF: Macrophage Colony Stimulating Factor

M-DM Monocyte-Derived Macrophages

miRNAs: MicroRNA

MITF: Microphthalmia-Associated Transcription Factor

MMP: Matrix Metallopeptidase

MOMP: Mitochondrial Outer Membrane Permeabilization

mRNA: messenger RNA

MTZ: Metronidazole

NF-κB: Rel/Nuclear Factor-κB

NRAS: Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog

NTR: Nitroreductase

Nu: Nude Nude Athymic

PBMCs: Peripheral Blood Mononuclear Cells

PDGF: Platelet-Derived Growth Factor

PDTXs: Patient-Derived Tumor Xenografts

PlGF: Placental-Derived Growth Factor

PMA: Phorbol-12-Myristate- 13-Acetate

PROTAC: Proteolysis Targeting Chimeric

PTEN: Phosphatase and Tensin Homolog

PUMA: P53-Upregulated Modulator of Apoptosis

RAF: Rapidly Accelerated Fibrosarcoma

RAS: Rat Sarcoma Virus

RBM: RNA Binding Motif Protein

RBPs: RNA Binding Proteins

RNA: Ribonucleic Acid

RNA Seq: RNA Sequencing

ROS: Reactive Oxygen Species

RRMs RNA Recognition Motifs

Comentado [VFMM1]: No entiendo lo que es

SAPK: Stress-Activated Protein Kinases

SC: Subcutaneously

SCID: Severe Combined Immune Deficiency

siRNA: Small Interfering RNA

SM: Spliceosomal

SMN: Survival Motor Neuron

SnRNP: Small Nuclear Ribonucleoprotein Polypeptide N

SRs: The Serine/Arginine-Rich

STAR: Signal Transduction and Activation of RNA

STAT: Signal Transducer and Activator of Transcription

T: Thymine

TAMs: Tumor Associated Macrophages

T-BID: Truncated Form of BH3-Interacting Domain Death Agonist

TGF: Transforming Growth Factor

TME: Tumor Microenvironment

TNF: Tumor Necrosis Factor

T-regs: Regulatory T Cells

UV: Ultraviolet

VEGF: Vascular Endothelial Growth Factor

VEGFR: VEGF Receptor

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SUMMARY

Melanoma represents the deadliest form of skin cancer. Features of melanoma are the poorly responsiveness and to standard chemotherapeutics and the high aggressiveness. Molecular mechanisms leading to melanoma development and progression are the focus of intense investigation, aimed at understanding its pathobiology and at developing new treatment strategies. A number of driver mutations have been identified and the most common mutations affect signaling of the Ras/Raf/mitogen- activated protein kinase pathways. In addition, the Bcl-2 family network is found deregulated in melanoma. Among Bcl-2 family proteins, B-cell lymphoma-extra Large (Bcl-xL) has emerged as multifaceted factor acting not only as a canonical anti-apoptotic factor, but also as a promoter of tumor progression. In melanoma Bcl-Xl has been found deregulated.

In the last years, the importance of the microenvironment for the tumor progression and response to therapy was established, underlying the importance of a continuous crosstalk between tumor cells and microenvironment, through secretion of tumorigenic mediators. In this scenario, tumor-associated macrophages (TAM) play a pivotal role affecting the nature of the tumor microenvironment. TAM infiltration is directly correlated to melanoma thickness and to increased angiogenesis and micro- vessel density, through modulation of tumor pro-inflammatory factors. Moreover, elevated number of TAMs in the tumor microenvironment is often correlated with poor prognosis in melanoma. Thus, macrophages represent promising therapeutic targets, and their depletion can represent an effective therapeutic intervention in the management of primary and metastatic melanoma. Macrophages can be classified in "classically activated macrophages" or M1, and "alternatively activated macrophages" or M2. TAM display an M2-like phenotype and are closely associated partners of malignant cells for

migration/invasion, angiogenesis, immune suppression, metastasis formation and drug resistance.

To prove the effect induced by the overexpression of Bcl-xL in the regulation of the tumor microenvironment human melanoma cells silencing or overexpressing Bcl-xL protein, THP-1 monocytic cells and monocyte-derived macrophages were used in this study. Protein array and specific neutralizing antibodies were used to analyse cytokines and chemokines secreted by melanoma cells. qRT-PCR, ELISA and Western Blot analyses were used to evaluate macrophage polarization markers and protein expression levels. Transwell chambers were used to evaluate migration of THP-1 and monocyte-derived macrophages. Mouse and zebrafish models were used to evaluate the ability of melanoma cells to recruit and polarize macrophages in vivo.

We demonstrated that melanoma cells overexpressing Bcl-xL recruit macrophages at the tumor site and induce a M2 phenotype. In addition, we identified that interleukin-8 and interleukin-1β cytokines are involved in macrophage polarization, and the chemokine CCL5/RANTES in the macrophage's recruitment at the tumor site. We also found that all these Bcl-xL-induced factors are regulated in a NF-κB dependent manner in human and zebrafish melanoma models. Our findings confirmed the pro-tumoral function of Bcl-xL in melanoma through its effects on macrophages demonstrating the ability of Bcl-xL to regulate macrophages polarization and recruitment via the secretion of specific factors, establishing tumor microenvironmental conditions that favour melanoma development.

INTRODUCTION

1. Melanoma

Melanoma is the most dangerous and deadly form of skin cancer representing more than 75% of all skin cancer deaths(1-4). Notwithstanding there have been new advances in the treatment of melanoma, prognosis remains poor, with a five-year survival rate of 16% for patients with distant metastases, and its incidence continue to increase (5). Causes associated with the hight mortality rate of melanoma are the aggressiveness the ability to early metastasize as well as the development of therapy resistance (6).

Furthermore, the incidence of malignant melanoma continues to rise year-over-year, with almost 106,000 new cases in the United States projected for 2022 (5) while in Italy in 2020 the estimated new cases of cutaneous melanoma were 100.350, representing approximately 5.6% of all cancer cases diagnosed in the same year, while the estimated deaths caused by melanoma were 97,610, representing 1.3% of all cancer deaths (https://seer.cancer.gov/statfacts/html/melan.html visited on 14 July 2023).

Melanoma originates from melanocytes, the pigment-producing cells present in the basal layer of the epidermis, usually when unrepaired DNA damage triggers mutations that lead the melanocytes to multiply rapidly and form malignant tumours. Melanocytes are present not only in the skin, but also in different other sites as in the mucous membrane generating the mucosal melanoma or in the eyes causing uveal melanoma or choroid melanoma or more rarely in the digestive tract. However, most frequently it affects the skin causing the cutaneous melanoma. Melanoma is caused mainly by intense exposure to ultraviolet radiation from sunshine or tanning beds (7, 8). According to that, UV causes a C>T substitution that prevail the genetic signature of cutaneous melanoma. Other factors, such as a

compromised immune system, or congenital risk factors as the amount of benign nevi, the skin type or the family history, could also cause melanoma (9). The transition from melanocytes to aggressive melanoma cells is caused by the accumulation of genetic alterations that could eventually lead to invasive form of melanomas(10).

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The Clark model explain the progression and development of melanoma: firstly, melanocytes proliferate forming a benign nevus. Although benign nevi rarely progress to cancer, some factors could affect the generation of dysplastic nevi. At this point melanomas have two growth stages: radial and vertical (11). During the radial growth stage, malignant cells grow in an outward movement, spreading across the epidermis. Eventually, most melanomas commence the vertical growth stage, characterize by the invasion of the dermis and the ability to expand or metastasize. The majority of first metastases are local affecting predominantly regional sites, including regional lymph nodes. The most common distant sites where melanoma spreads include lung, liver, distant areas of the skin, brain, gastrointestinal tract, as well as bone and adrenal gland (Figure 1) (12, 13).

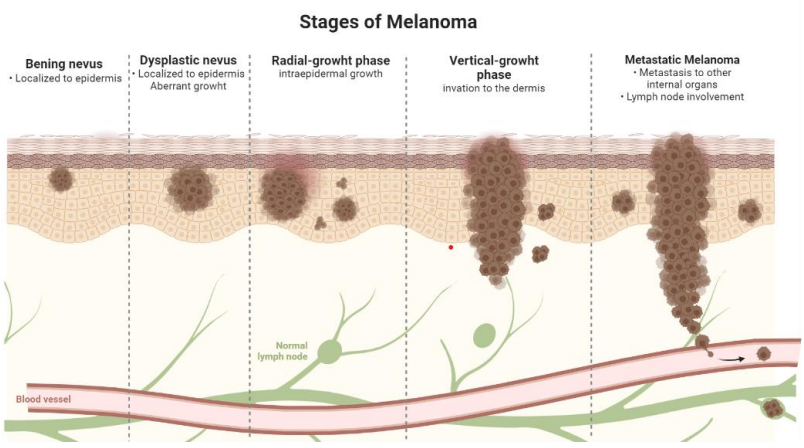

Figure 1 Melanoma's stage: the development of melanoma is a multistep process, that begins with the generation of benign nevus and ends metastasizing. During the radial phase melanocytes spread in the epidermal layer, acquiring then the ability to spread and invade the dermis during the vertical phase. Finally, cells acquire the ability to invade distant organs via blood and lymph vessels. Illustration adapted from "Melanoma Staging", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

The stepwise transformation from melanocytes to melanoma cells is due to the accumulation of mutations in several oncogenes and tumor suppressor genes. The constitutive activation of the RAS-RAF-MAPK/ERK pathway, is often associated with the development of melanoma. This pathway controls cell proliferation, invasion, angiogenesis, and metastasis (14, 15). Often, v-raf murine sarcoma viral oncogene homolog B (BRAF) and the neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) are constitutively activated causing a permanent signaling of the mitogen-activated protein kinase (MAPK) pathway and thus and uncontrolled proliferation of the cells (16) (Figure 2).

Notably, melanocyte-specific expression of BRAFV600E protein consistently leads to benign melanocytic lesions, indicating that additional events are required for melanocyte transformation.

Microphthalmia-associated transcription factor (MITF) is the master transcription factor regulating proliferation and differentiation of melanocytes and has also been associated with melanoma (17). The role of MITF is complex: while melanoma cells expressing MITF at a high level can either differentiate or proliferate, low activity of MITF is related to stem cell-like or invasive potential (18).

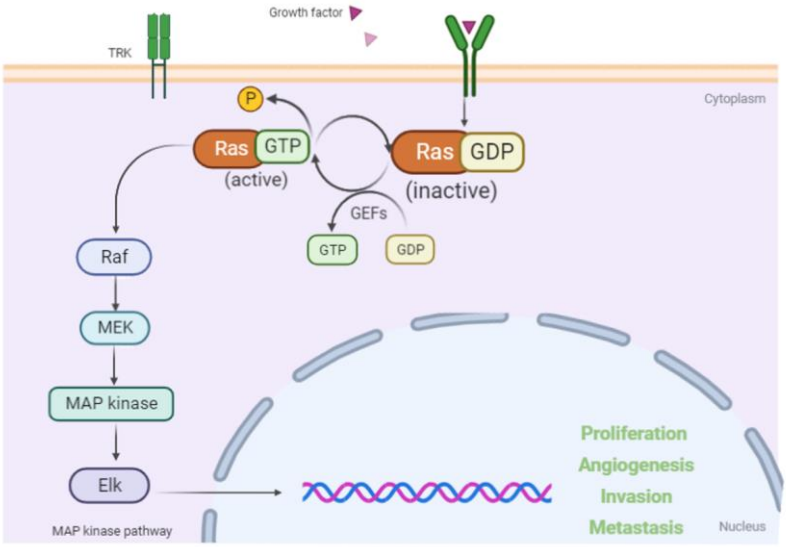

Figure 2: MAPK pathway: the constitutive activation of NRAS or BRAF causes a constitutive signaling causing an uncontrolled proliferation of melanoma cells. Illustration adapted from "Ras Activation", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates. Retrieved from https://app.biorender.com/biorender-templates.

One of the main problems for the treatment of melanoma is its heterogeneity associated with its ability to metastasize to different organs, making the latest treatments improved in the last years effective for patients in early stages, but an overall poor survival for patients with advanced cancers.

Recently, different therapies have been approved by the US Food and Drug Administration (FDA) for melanoma treatment (19-21). Depending on the features of the tumor (e.g. location, stage, genetic profile), the therapeutic protocol may require surgical resection, chemotherapy, radiotherapy, photodynamic therapy, target therapy and immunotherapy. Surgery is the main course of treatment for people with stage I-IIIB melanoma. (21). Surgery is often associated with the use of chemotherapy, although therapeutic treatment could include side effects due to the toxicity at the level of the skin or gastrointestinal tract, as well as immune reactions and reduced efficiency, which can occur as resistance to chemo- or intra-lesion therapies due to the heterogeneity of the tumor (22). New therapeutic targets have been described from genetic studies on melanocytes and from the identification of factors involved in the pathogenesis of the malignant transformation of the melanocytic cells (23-25). All these studies pointed out the importance of investigating how those mechanisms may contribute to melanoma pathogenesis and progression.

2. Apoptotic pathways

Throughout their evolution, multicellular organisms have developed the ability to eliminate damaged or unwanted cells with the aim to maintain a continuous homeostasis of tissues. This constant turnover is essential to the development of healthy tissues and organisms. Cells are eliminated through regulated cell death mechanisms including apoptosis, necrosis, and autophagy (26). These mechanisms have been developed in all the vertebrates and have preserved well with evolution, securing an orderly development of organisms (27, 28) whilst preventing the onset of diseases, including cancer (29, 30). Specifically, impaired apoptosis represents a hallmark of cancer (31). The apoptotic pathways have at the basis of the process the activation of caspases, that degrade cellular components in order to be recognized by phagocytes (32, 33). Caspases are part of a family of cysteine proteases preserved throughout evolution and involved in cell death, as well as in the inflammatory response (34, 35). The apoptotic caspases can be divided into "initiator" (caspases 8, 9, 10) and "effector" (caspases 3, 6, 7) caspases (35). Under physiological conditions, caspases are present in the form of inactive pro-caspases, which are activated in a cascade process that ends up with the activation of the effector caspases through proteolytic cleavage and consequent induction of cell death (36-38). For such process to take place, the cell must receive a series of external or internal stimuli that activate the apoptotic pathway. Two different pathways in the activation of this process are known to date, thereof one is commonly defined as "intrinsic" and mediated by mitochondria, and the other one is known as "extrinsic" and mediated by death receptors (39). The extrinsic pathway is induced by death ligands produced by patrolling cells, and for this reason, it is also known as the death receptor pathway of apoptosis (40). After death ligand binding to the death receptors expressed in the target cell membrane, the extrinsic pathway is induced through the activation of caspase 8 (26). In contrast, the intrinsic process can be induced by all cells in response to various stress stimuli:

examples include oxidative and endoplasmic reticulum stress, nutrient deprivation, pathogen infection or DNA damage (Figure 6) (41). The key regulators of this process are members of the B-cell lymphoma 2 (Bcl-2) family which, following activation, cause at first a permeabilization of the mitochondrial outer membrane, and then a release of cytochrome C from the mitochondria into cytosol, assembly of the apoptotic protease activating factor-1 (APAF-1) forming apoptosome by oligomerization and at last, activation of the caspase cascade through caspases 9 (41, 42). A strong correlation between incorrect apoptotic process and various pathologies has been identified (43). Tumor cells, for example, increase their pro-survival activity during the tumorigenic process, thus developing resistance to apoptotic stimuli (44).

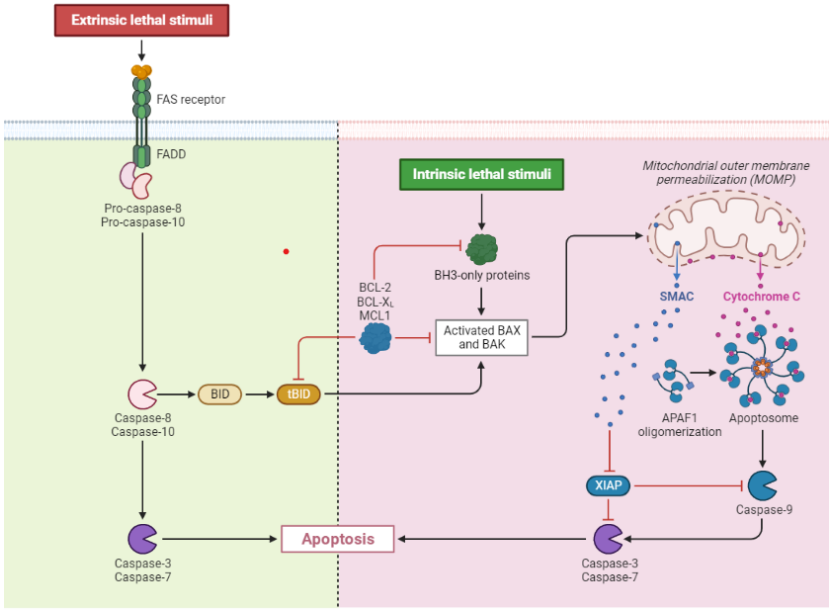

***Figure 3 Schematic representation of the apoptotic pathways*:** The extrinsic pathway involved the production of death ligands by patrolling cells. The extrinsic pathway can be induced by all cells in response to various stress stimuli. Illustration adapted from "Apoptosis Extrinsic and Intrinsic Pathways", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

2.1 Bcl-2 Family

Proteins of the Bcl-2 family are essential supplements to the signals leading to cell survival or apoptosis, while the cell's fate itself depends primarily on quantity, location and interaction among specific Bcl-2 family proteins(45). Members of the Bcl-2 family are classified according to structure

and function. The anti-apoptotic members of this family, which include Bcl-2, B-cell lymphoma-extralarge (Bcl-xL), B-cell lymphoma-w (Bcl-w), Bcl-2-related protein A1/Bcl-2-related isolated from fetal liver-11 (A1/Bfl-1) and myeloid cell leukemia-1 (Mcl-1) share 4 Bcl-2 homology domains (BH1-BH4). The peculiar trait shown in this group is the presence of the BH4 domain in the N-terminal (Figure 7) (46). Through the BH4 domain, Bcl-xL binds the pro-apoptotic proteins of the Bcl-2 family, such as Bcl-2-Associated X protein (BAX) and Bcl-2 Associated Agonist Of Cell Death (BAD), thereby preventing the activation of apoptotic signaling that leads to the opening of pro-apoptotic ion channels(47). By doing so, Bcl-xL supports cell survival by inhibiting intrinsic cell death pathway, such as the release of cytochrome C and apoptosome assembly (48, 49). In the case of Bcl-2, when a mutation on the BH4 domain occurs, this reduces the stability of the protein and interferes with the occupation of BAX (50).

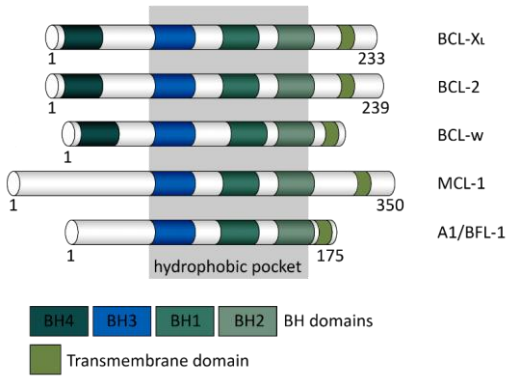

Figure 4 Schematic representation of the anti-apoptotic Bcl-2 family members. Bcl-2 homology (BH) and transmembrane domains are represented in distinct colours. The hydrophobic pocket is marked in grey. Lucianò, Anna Maria et al. "Bcl-xL: A Focus on Melanoma Pathobiology." International journal of molecular sciences vol. 22,5 2777. 9 Mar. 2021, doi:10.3390/ijms22052777

As the BH4 domain is capable of binding to other proteins that do not belong to the Bcl-2 family, some anti-apoptotic members, including Bcl-xL, are capable of more than just inhibition of apoptosis to which are traditionally associated, specifically contributing to other important cellular functions, such as proliferation, autophagy, differentiation, DNA repair, tumor progression and angiogenesis (51). Regarding their pro-survival role, anti-apoptotic proteins of the Bcl-2 family discharge their duties by binding and inhibiting pro-apoptotic proteins, cell stress sensors (proteins BH3-only) and apoptosis effectors, BAX and Bcl-2 antagonist/killer (BAK) (45). The pro-apoptotic proteins that belong to the Bcl-2 sub-group like BAX and BAK share the BH1-BH3 domains (52). The third class of proteins possesses the BH3 domain: Bcl-2-interacting mediator of cell death (BIM), p53-upregulated modulator of apoptosis (PUMA) and truncated form of BH3-interacting domain death agonist (tBID)(53). These proteins are called “activators”, as they can bind and provoke new conformations of BAX or BAK to induce mitochondrial outer membrane permeabilization (MOMP), while BH3 proteins that do not associate with BAX and BAK are called “synthesizers”, like BAD or NOXA that endorse apoptosis by both directly activating BAX and BAK and by suppressing the anti-apoptotic proteins at the mitochondria and the endoplasmic reticulum (53-55). The perfect functioning of these systems lies in the delicate balance between the different protein components of the Bcl-2 family: in particular, in the activity of (i) the anti-apoptotic proteins that retain the activators and the molecules that initiate MOMP, and (ii) the sensitizers, which antagonize members of the pro-survival family by releasing the only BH3-activating proteins BAX/BAK.

2.2 Bcl-xL: The Pro-Survival Member of the Bcl-2 Family

Bcl-xL is encoded by the *BCL2L1* gene, which is located on human chromosome 20 at band q11.21 and gives two isoforms of mature RNA through alternative splicing: the short form Bcl-xS, containing three exons, and the large form Bcl-xL, containing 4 exons (56). At protein level, three different isoforms can be found: the longer form, containing 233 amino acid residues in length, acts as an apoptotic inhibitor; the shorter form, of 170 amino acid, acts as an activator of apoptosis; and a third form, called Bcl-x, containing 227 amino acids, differs from the longer and shorter forms in the last 45 amino acids, but does not display any known function (Figure 8) (57).

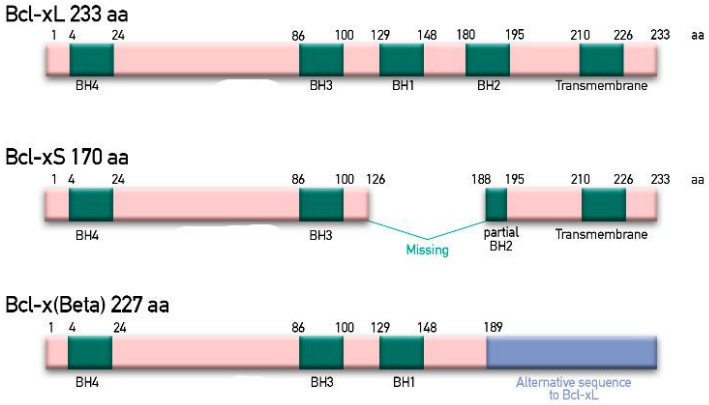

Figure 5 Schematic representation of the alternative spliced variants Bcl-xL, Bcl-xS and Bcl-x(Beta). Bcl-2 homology (BH) and transmembrane domains are represented in green. Alternative sequence present in Bcl-x(Beta)(Bcl-x) is reported in violet. No defined domains are shown in pink. Lucianò, Anna Maria et al. "Bcl-xL: A Focus on Melanoma Pathobiology." International journal of molecular sciences vol. 22,5 2777. 9 Mar. 2021, doi:10.3390/ijms22052777

Bcl-xL protein structure is made up of 8 α helix regions. A5 and α6 integrate a central hairpin that is flanked by the α3 and α4 on one side, and by α1, α2 and α8 on the other side (58). Looking at their three-dimensional structures, BH domains appear essential to the formation of tertiary structures. The BH1 and BH2 domains cover the regions connecting each two helices, α1 and α2 in the case of the BH1, and α7 and α8 in the case of the BH2(58). The BH3 domain is located entirely in the α2 helix, while the BH4 domain is located in the α1 and makes up hydrophobic contacts with α2, α5 and α6(58).

A rather interesting structural element of Bcl-xL is a large hydrophobic groove involving the BH1 and BH3 domains, predominantly formed between the α3 and α4 helices (Glutamine-111 on α3 and Glutamic acid-129 on α4), and the α5 found at its basis. This region is deemed to be the most relevant difference among members of the pro-survival protein family, which seems to have the same conformation and little identity of sequence. In fact, other members have a v-shape structure of the helices resulting in a more open groove (59-62).

The finely regulated interaction between pro- and anti-apoptotic proteins is made possible by the spatial architecture of the BH3 domains. In fact, the hydrophobic groove appears to be responsible for the capture of the pro-apoptotic molecules. In most of the cases, the overall interaction is determined by 4 hydrophobic residues of the BH3 domain projecting into the groove and an electrostatic interaction between the conserved aspartate- 83 and arginine-139 in Bcl-xL (63, 64). After interacting with the BH3 domain, the immediate reaction is an opening of the hydrophobic groove where the α3 shifts away and the α4 moves to create an opening of the groove that acquires a V-shaped form. This phenomenon is typical of Bcl-xL protein and sets it apart from other family members (65).

Bcl-xL is important for the p53-induced permeabilization of the mitochondria and apoptosis because of its interaction with the DNA-binding domain of the p53 protein. Nuclear magnetic resonance spectroscopy identified the carboxy-terminus of the first α-helix, and the loops between α3/α4 and α5/α6 as the Bcl-xL regions involved in the binding with p53 (66-68).

2.3 Regulation of Bcl-xL

The *BCL2L1* promoter is highly conserved between human and mouse. When isolated for the first time, both murine and human promoters have shown two distinct regions with promoter activity (69).

The first promoter region is located immediately above the first codon exon, while the second one is above the first non-coding region (70). Moreover, consensus motifs for a number of transcription factors have been identified, as Sp1, AP1, Oct-1, E26 transformation specific (ETS), Rel/Nuclear Factor-κB (NF-κB), Signal Transducer and Activator of Transcription (STATs), and GATA-1, among which STATs, Rel/NF-κB, ETS and the AP1 complex have been demonstrated to play an important role in the regulation of *BCL2L1* gene expression (Figure 9) (56, 69).

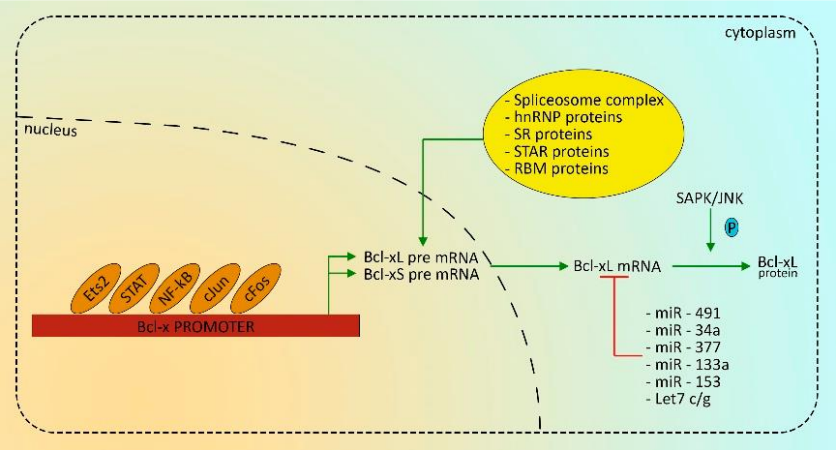

Figure 6 Transcriptional factors and effectors involved in the Bcl-xL regulation. Lucianò, Anna Maria et al. "Bcl-xL: A Focus on Melanoma Pathobiology." International journal of molecular sciences vol. 22,5 2777. 9 Mar. 2021, doi:10.3390/ijms22052777

A list of putative transcription factors able to bind the *BCL2L1* promoter identified via the UCSC Genome browser (https://genome.ucsc.edu/ accessed on 15 February 2021) (71) shows the list that we cross-checked using the Lasagna tool browser (https://biogrid-lasagna.engr.uconn.edu/lasagna_search/index.php accessed on 15 February 2021). Among these, only few transcription factors have been confirmed to be able to bind Bcl-xL promoter and/or to regulate Bcl-xL expression. The first transcription factor identified in that respect was ETS2, belonging to the family of ETS. It was revealed by a specific co-expression of ETS2 and Bcl-xL in the same population of developing T-cells (72). ETS2/Bcl-xL co-expression has been also identified in CD4+/CD8+ T cells, in primary bone marrow macrophages (BMM) derived from bone marrow progenitor cells, and in BMM upon activation of functional competence signals (73). ETS2 was able to upregulate Bcl-xL protein expression when produced in macrophages or human embryonic kidney cells, making these cells resistant to apoptosis (72).

NF-κB was found to upregulate *BCL2L1* expression, influencing its expression as evidenced for CD40 survival signals in human B lymphoma cells (74). Moreover, c-Rel, a subunit of the NF-κB family, has been reported to be responsible for Bcl-xL expression. These results demonstrate that some death antagonists of the Bcl-2 family can affect oncogenesis through the effect of NF-κB on their expression(75).

The STAT family is activated after phosphorylation by JAKs and acts as a downstream effector of different cytokines or growth factors (76). Different results have reported the involvement of several members of the STAT family in the transcriptional regulation of the BCL2L1 gene, both in normal cells, such as hematopoietic and myoblast cells, and cancer cells. On the one hand, the first involvement of STAT3 in the regulation of Bcl-xL has been proved by Grandis and colleagues throughout the identification of a correlation between the constitutive activation of STAT3 and a

higher expression of Bcl-xL in squamous cell carcinomas of the head and neck (77). On the other hand, a reduced expression of STAT3 caused a decreased *BCL2L1* expression and induced apoptosis, while a functional STAT3 was necessary for *BCL2L1* reporter gene activity in myeloma cells, suggesting that STAT3 activity underlies the high expression of Bcl-xL detected in these tumors (78). A positive correlation between *BCL2L1* expression and phosphorylated STAT3 was also identified in melanoma models (79). In physiological conditions, the regulation of *BCL2L1* by STAT5 has been proved in hematopoietic cells (80). Studies showing that STAT1, but not STAT3, mediated the expression of the *BCL2L1* gene upon treatment of cardiac myoblasts with leukemia inhibitory factor were also reported(81).

c-Fos and c-Jun are also involved in the regulation of *BCL2L1*. Jun proteins can homo-dimerize, whilst Fos proteins can only hetero-dimerize. They can form a complex defined as AP1 and regulate the transcription of target genes, whose promoter contains the AP1 binding sites. Further, the AP1 complex was found to affect both proliferation and differentiation pathways (82). AP1 activity has been dubbed potentially crucial in controlling anti-apoptotic genes (83). Presence of an AP1 site upstream of the first non- coding exon has been detected on the *BCL2L1* promoter, and c-Fos and c-Jun have been shown to be able to trans-activate the *BCL2L1* gene(72).

The splicing control of *BCL2L1* is central to regulate apoptotic response in normal development. As previously stated, *BCL2L1* gene can be alternatively spliced to produce the long-form Bcl-xL and the short-form Bcl-xS. Because of their different roles, the regulatory mechanisms of *BCL2L1* splicing have been largely investigated. A study conducted by Moore and coworkers (84) identified several components of the spliceosome complex that are directly involved in *BCL2L1* splicing. The study highlighted the fundamental role played by the alternative splicing network to regulate cell cycle control under normal and mitotic stress (84). Specifically, the authors identified Small Nuclear

Ribonucleoprotein Polypeptide N (snRNP) and splicing factors including U1 snRNP protein (SNRP70); U2 snRNP proteins (SF3B1, SF3B4, SF3B5, SF3A1, SF3A3, SNRNPA1); U5 snRNP proteins (U5-200K, PRPF6, UPS39); spliceosomal (Sm) and like Sm (LSM) core proteins (SmB/B′, SmD1, SmD2, SmD3); survival motor neuron (SMN) complex proteins (SMN1, GEMIN4: Gem-associated protein 4) (85).

Studies conducted in the last decade have identified also other RNA binding proteins that enhance or inhibit *BCL2L1* splicing, with consequent effect on apoptosis. These factors have been classified in 4 families (85):

i. Heterogeneous nuclear ribonucleoproteins (hnRNP) that are a large family of RNA binding proteins (RBPs) that control multiple processes in RNA metabolism and whose function depends on their ability to bind the RNA and their localization. Six different hnRNP regulating *BCL2L1* splicing have been identified. Among them, four have been reported to promote the production of Bcl-xS and two the production of Bcl-xL (86).

ii. The serine/arginine-rich (SR) protein family that is a group of RBPs that contains a characteristic C-terminal arginine and serine-rich domain (RS domain) and one or two N-terminal RNA recognition motifs (RRMs) (87). Different SR proteins have been identified as a key regulator in the splicing of BCL2L1. The most important of these proteins is the SRF10, which is involved in DNA damage, apoptosis, DNA repair and it has been shown to promote Bcl-xS production (88).

iii. Signal transduction and activation of RNA (STAR) proteins, which constitute a family with a conserved K homology-domain and a STAR domain, responsible for the RNA recognition(89, 90). The STAR proteins can regulate the metabolism of the RNA, including the splicing. To date, the only STAR protein involved in the regulation of *BCL2L1* splicing is SAM68, that when overexpressed is able to increment the isoform Bcl-xS, whilst when depleted induces an accumulation of Bcl-xL(91).

iv. The RNA binding proteins, which possess the RRMs (92). Within this group of proteins, the RBM25 has been shown to specifically interact with a sequence in exon 2 of BCL2L1 promoting the pro-apoptotic Bcl-xS 5′ splice site selection(93), while RBM11 plays an important role in the regulation of alternative splicing for neuron and germ cell differentiation(92). RBM4 performs important functions in the inhibition of tumor progression. Specifically, it induces cancer cell apoptosis by modulating *BCL2L1* splicing and shifting to the pro-apoptotic Bcl-xS isoform. Additionally, RBM4 can also antagonize the oncogenic SR protein SRSF1 to regulate *BCL2L1* splicing and inhibit cancer cell growth(94).

Finally, in the last decades, different microRNAs (miRNAs) were found to target and decrease the expression of the anti-apoptotic Bcl-2 family members(85). To date, most miRNAs identified are able to target only one of the anti-apoptotic family members even if the list of miRNAs capable of targeting different members increases over time. At least we can count three different miRNAs able to target different members of the Bcl-2 family: the miR-125b, whose targets are Bcl-2, Mcl-1 and Bcl-w (95, 96), miR-133a/b able to target Mcl-1 and Bcl-xL (97), and miR-153 that targets Bcl-2 and Mcl-1 (98). Among the miRNAs able to act against Bcl-xL we cite let-7c/g, which negatively regulates Bcl-xL expression (99); miR-491 which is able to induce apoptosis by targeting Bcl-xL in different tumor types such as glioblastoma, ovarian and colorectal cancer (100-102); miR-34a that is able to target Bcl-xL (103); and miR377 that represses Bcl-xL (104).

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Other modifications that Bcl-xL protein can undergo are phosphorylation mediated by SAPK/JNK causing an inhibition of *BCL2L1* expression (105, 106), as it has been demonstrated for Bcl-2 and Mcl-1 (107).

2.4 Bcl-xL expression and canonical functions in melanoma

As previously discussed, the apoptotic process is unquestionably a necessary tool for tissue regeneration and long-lasting health, but at the same time, its alteration has been a strategy widely exploited by cancer cells as a defence mechanism against multiple therapies. In fact, tumor cells are able to escape apoptosis, ensuring an adaptation to the microenvironment and therapies. Melanoma cells feature a distinctive labile and stage-dependent phenotype, which is why pro-survival molecules can protect them from apoptosis and mediate other processes, thus increasing an aggressive phenotype.

By analysis of samples from human benign nevi, primary melanoma, and melanoma metastases in comparison with normal skin, the study of Leiter and colleagues demonstrated that Bcl-xL was expressed in all metastatic melanoma samples, 80% of nevi and 62% of normal tissue samples, illustrating how Bcl-xL increases its expression passing from primary to metastatic melanoma (108). The involvement of Bcl-xL in melanoma progression was also proved by Zhuang's group via immunohistology analysis of Bcl-xL and other anti-apoptotic proteins, as Bcl-2 and Mcl-1, in sections of benign nevi, primary melanoma and metastatic melanoma (79). Even if Bcl-xL was expressed in benign nevi and thin melanoma, its expression was higher in sub-cutaneous and lymph node metastases when compared to benign nevi and thin melanoma. A positive correlation between Bcl-xL and tumor thickness or mitotic rate was also evidenced. An enhanced expression of Bcl-xL and Bcl-2 proteins passing from primary to metastatic melanoma has also been shown by Zhang and colleagues (109). By using primary cell cultures derived from melanoma specimens, established cell lines and normal melanocytes from healthy donors, high Bcl-xL expression was observed in all melanoma samples tested (110). Interestingly, the expression of the pro-apoptotic Bcl-xS isoform has been reported to decrease during melanoma progression (108). From all these findings, it is evident

that elevated expression of Bcl-xL is associated with melanoma progression from primary into metastatic melanoma.

The canonical role of Bcl-xL as an anti-apoptotic factor in melanoma was fully elucidated in a recent study (111), with the aim of evaluating the role that the different components of the Bcl-2 family play in melanoma. The authors conducted a study testing different BH3 mimetic drugs designed to target individuals or sub-groups of pro-survival Bcl-2 proteins, alone and in combination, in both 2D and 3D cell cultures in a panel of established and early-transition patient-derived cell lines. The study demonstrated that none of the drugs showed significant effects on a stand-alone basis, while combinations of drugs targeting Mcl-1 and Bcl-xL had a synergistic ability to kill cancer cells, hence providing evidence of how Bcl-xL and Mcl-1 appear to be key factors in maintaining melanoma cell survival. This study also shows a clear dissociation between changes in Bcl-2 expression (downregulation) and Bcl-xL or Mcl-1 expression (upregulation) during progression of melanoma. A different involvement of Bcl-xL and Bcl-2 was also reported in the study of Zhang and Rosdahl, indicating that Bcl-xL, but not Bcl-2, was a key protein in the induction of apoptosis after ultraviolet-B exposure in melanoma cells (109). On the contrary, the study of Olie and collaborators performed using antisense oligonucleotides directed against either Bcl-xL mRNA or the Bcl-2 and the Bcl-xL mRNAs simultaneously (110) demonstrated that both the Bcl-xL monospecific oligonucleotide and the Bcl-2/Bcl-xL bispecific oligonucleotide reduced tumor cell viability by induction of apoptosis, but the bispecific oligonucleotide proved to be superior to the monospecific ones.

Although there are no clear explanations of how Bcl-xL could confer chemo-resistance, the aggressiveness induced by an over-expression of Bcl-xL has been often associated to its capacity to induce drug resistance in cancer from different origins (111-114). Regarding melanoma, it has been demonstrated that forced expression of ectopic Bcl-xL converted drug-sensitive cell lines into drug-

resistant ones. Bcl-xL contributes to melanoma chemo-resistance through the protection from drug-induced apoptosis (114, 115). Reduction of Bcl-xL protein expression by specific antisense oligonucleotide enhanced the chemo-sensitivity of melanoma cells as well as chemotherapy-induced apoptosis. These data suggest that Bcl-xL is an important factor contributing to the chemo-resistance of human melanoma and can be inhibited by antisense therapy. Bcl-xL's ability to induce chemo-resistance has also been attributed to the interaction between Bcl-xL and the Insulin Growth Factor (IGF1) (116). Further, the specific silencing of both Bcl-xL and IGF1 by small interfering RNA evidenced the protective effect of IGF1 and a correlation with STAT5, suggesting promotion of anti-apoptotic chemo-resistance mechanism via the activation of the anti-apoptotic protein Bcl-xL(116). Furthermore, the overexpression of Bcl-xL also blocked the cytochrome C release induced by anticancer drug, promoting cell drugs resistance and survival (117). Finally, a downregulation of Bcl-xL after ultraviolet B radiation has been reported in primary melanoma samples, whereas matched metastatic specimens expressing higher Bcl-xL level were not affected by the treatment (109).

2.5 Non-canonical functions of Bcl-xL in melanoma

In addition to its well-known role in canonical pathways such as apoptosis, and survival in cancer, Bcl-xL also affects other pathways, independent from survival and apoptosis, such as invasion/migration, epithelial mesenchymal transition, metastasization, and stemness (Figure 4). These biological functions have been observed in tumours from different origin, such as glioma, breast, colorectal and pancreatic cancer (118-121). In some cases, the role of Bcl-xL in the nucleus, but not in the mitochondria, has been identified as responsible for its non-canonical functions (118). Moreover, the non-canonical functions of Bcl-xL have been reported by our group in melanoma. Recently, we have demonstrated that Bcl-xL incremented the in vitro cell migration and invasion in melanoma models, facilitating at the same time the formation of a vasculogenic structure (122).

Analysis of Bcl-xL overexpressing cells proved their ability to form tumor spheres associated to a pattern of stemness markers, supporting the idea that Bcl-xL is also important for the maintenance of cancer stem cell phenotype. In the past years, we focused our attention on the role of Bcl-xL in the regulation of angiogenesis in melanoma models. We found that conditioned medium of Bcl-xL overexpressing cells increased in vitro endothelial cell functions and in vivo vessel formation through the pro-angiogenic factor interleukin-8 (IL-8, also known as CXCL8). The use of neutralizing antibodies against IL-8 confirmed the role of this chemokine in the angiogenesis induced by Bcl-xL overexpression. Downregulating Bcl-xL through antisense oligonucleotide or siRNA, confirmed the involvement of Bcl-xL in the expression of IL-8 both at the protein and mRNA level. When melanoma cells were grown under low oxygen condition (hypoxia), we also highlighted Bcl-xL ability to increase the expression of the hypoxia inducible factor (HIF-1) and its target genes, vascular endothelial growth factor (VEGF) and metalloproteases 2 (123). In fact, although in normoxic condition Bcl-xL did not affect the HIF-1/VEGF pathway, under hypoxia Bcl-xL, overexpression resulted in a higher level of HIF-1α and VEGF (122). These data are consistent with the ability of Bcl-2 protein to cooperate with hypoxia to induce angiogenesis through VEGF in cancer models (122, 124). All these results corroborated the previous reported findings obtained in vitro and in vivo showing a unidirectional crosstalk able to empower the angiogenic phenotype of the endothelial cells (125). In particular, in endothelial cells Bcl-xL was able to promote the release of VEGF, which after binding with its receptor VEGFR induced the expression of Bcl-2 and consequent induction of interleukin-1 and IL-8, and activation of the autocrine signaling pathways enhancing the angiogenic activity.

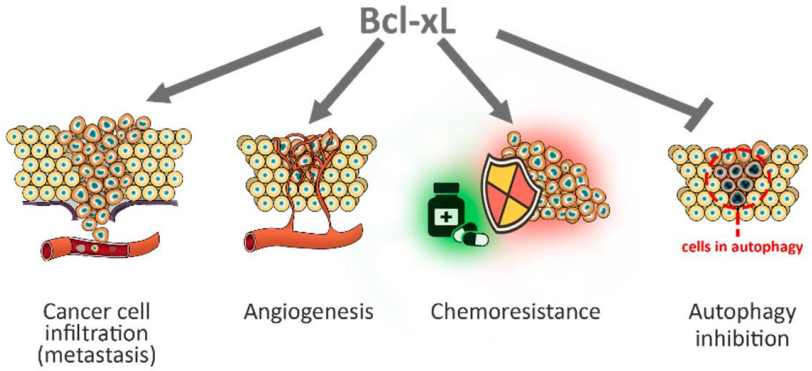

Figure 7 Non-canonical role played by Bcl-xL on melanoma. Bcl-xL is involved in metastasis, angiogenesis, chemoresistance against different chemotherapeutic drugs, and autophagy. Lucianò, Anna Maria et al. "Bcl-xL: A Focus on Melanoma Pathobiology." International journal of molecular sciences vol. 22,5 2777. 9 Mar. 2021, doi:10.3390/ijms22052777

To further investigate the involvement of Bcl-xL in angiogenesis, was also analyzed whether the Bcl-xL/IL-8 pathway was important to promote angiogenesis and aggressiveness in zebrafish melanoma models (126). Bcl-xL overexpressing melanoma cells showed enhanced dissemination and higher angiogenic activity in zebrafish embryos. Human IL-8 protein was also able to induce a strong pro-angiogenic activity in zebrafish embryos, and using a morpholino-mediated gene knockdown, the CXCR2 receptor was identified as the mediator of IL-8 pro-angiogenic activity. What stood out from our results was how aggressiveness of melanoma cells overexpressing Bcl-xL was mediated by an autocrine effect of IL-8 on its receptor. Finally, via microarray and RNA seq public databases, a

correlation between IL-8 and markers of melanoma aggressiveness was also identified, together with a correlation between Bcl-xL and IL-8 expression and poor prognosis of melanoma patients (126).

Lastly, Bcl-xL results involved also in autophagy, a process by which damaged or old cells undergo degradation (127). Just as apoptosis, autophagy is essential to maintain tissue homeostasis but, at the same time, a deregulated process is often observed in pathological conditions, including tumors (128, 129). Although apoptosis and autophagy occur for different reasons, they share common stimuli as well as regulatory proteins, including p53, beclin1 and the Bcl-2 family. Several studies have demonstrated the ability of Bcl-xL to bind beclin1, a key regulatory factor indispensable for the formation of the autophagic complex (130). In particular, the binding of these two proteins determines an inhibition of autophagy, thanks to the presence of a BH3 domain on beclin1, which can bind the hydrophobic groove of Bcl-xL (131). It is particularly interesting how Bcl-xL under normal conditions protects cells from autophagy by inhibiting the Beclin-1-Vps34 complex, while in cancer cells induces autophagy by interacting with mitochondrial ARF tumor suppressor. As suggested by the authors, a possible explanation is that when the expression of ARF protein increases, Bcl-xL, binds ARF, thus leaving free Beclin-1-Vps34 complex to act (132).

2.6 Preclinical studies of Bcl-xL: Specific Inhibitors on Melanoma

Because of its relevance in the progression of cancer from different histotypes, different strategies have been evaluated in order to inhibit Bcl-xL. These include antisense oligonucleotides, small molecules including BH3 mimetics, Proteolysis Targeting Chimeric (PROTAC) molecules.

The first approach used to inhibit Bcl-xL was the development of antisense oligonucleotide targeting the mRNA of Bcl-xL or the mRNA of different antiapoptotic proteins. Has been previously demonstrated that antisense oligonucleotides against both Bcl-xL and Bcl-2 were able to inhibit

angiogenesis in melanoma cell lines overexpressing Bcl-2 (133). The same effect was also confirmed by Olie and colleagues, who used antisense oligonucleotides targeting either the Bcl-xL mRNA or the Bcl-2 and the Bcl-xL mRNA on primary cell cultures, stable cell lines and normal melanocytes from healthy donors (112, 134). They proved that both Bcl-xL monospecific oligonucleotide and Bcl-xL/Bcl-2 bispecific oligonucleotide effectively reduced tumor cell viability by induction of apoptosis. Bcl-xL/Bcl-2 bispecific antisense oligonucleotide also triggered p53-independent apoptosis in human melanoma cells (135). Interestingly, the strategy with antisense oligonucleotide was successfully applied also in vitro and in vivo in combination with cisplatin in tumor models from different origin not including melanoma, such as mesothelioma (136), transitional cell carcinoma (137) and bladder carcinoma (138).

Another strategy used to inhibit Bcl-xL and other anti-apoptotic proteins is represented by small molecules able to abrogate the expression of anti-apoptotic members, mimicking the action of the BH3-only proteins. Initially, the formulation of these BH3 mimetic was against all the Bcl-2 anti-apoptotic members. One of the first small molecules used in melanoma was the pan BH3 inhibitor obatoclax (GX15-070), showing affinity for Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1/Bfl-1(139). Another small molecule inhibiting Bcl-2, Bcl- xL and Bcl-w is represented by ABT-737, whose effect on melanoma was demonstrated because of its ability to empower the efficacy of several therapeutic strategies including immunotoxins (140) and BRAF or MEK inhibitor in BRAF-mutated cells (141). As it is not orally available, ABT-737 was replaced by ABT-263 (Navitoclax). The efficacy of Navitoclax has been reported for the in vivo treatment of BRAFV600E melanoma models in combination with copper chelators, able to sequester copper required for MEK1 and MEK2 activity through a direct copper–MEK1/2 interaction(142). Unfortunately, the use of Navitoclax was restricted because of the side effect that it caused (143). Nevertheless, a phase I/II trial study using Navitoclax in combination with dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) is

recruiting unresectable or metastatic patients with BRAF mutant melanoma (ClinicalTrials.gov, NCT01989585).

The first BH3 mimetic small molecule described to act specifically on Bcl-xL has been WEHI-539 (144). It was identified by structure-guided design of selective Bcl-xL inhibitors and showed more than 400-fold selectivity for Bcl-xL when compared to other Bcl-2 antiapoptotic family members (145). WEHI-539 induced a synergistic effect in melanoma models when combined with mitochondrial chaperone, G-TPP (146). Its use was limited because of the poor physicochemical properties and high in vivo toxicity (144, 147).

In recent times, the formulation of the first-in-class Bcl-xL inhibitor, A-1331852, has been described. It was produced by re-engineering BH3 mimetic Bcl-xL inhibitor, A- 1155463, using structure-based drug design. A-1331852 is an orally active compound able to induce apoptosis in Bcl-xL-dependent tumor cells (148). Unfortunately, there are still no preclinical tests able to provide efficiency of A-1331852 treatment in melanoma. Regarding A-1155463, while it did not affect melanoma cell viability, it increased the effect of immunotoxin targeting the melanoma-associated chondroitin sulfate proteoglycan 4 (CSPG4) when combined with the Mcl-1 specific inhibitor, S63845(140)

Recently, DT2216, a Bcl-xL PROteolysis-TArgeting Chimera (PROTAC) derived from ABT-263, has been reported to be a potent inhibitor of different forms of cancer and with low toxicity versus platelets. The effect of DT2216 was also demonstrated *in vivo* where the outcome was proved both as a single agent and in combination with chemotherapeutic drugs (149). No data are available regarding the effect of this chimera in melanoma model**s.**

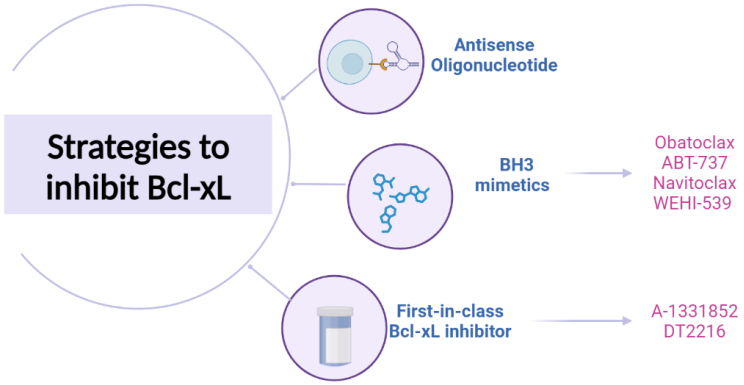

Figure 8 Strategies to inhibit Bcl-xL: Created with BioRender.com

3 Tumor Microenvironment

To progress and metastasize, the tumor needs to maintain a continuous crosstalk with the surroundings, generating the so-called tumor microenvironment (TME). Even if the progression of cancer has been always described as a multiple steps process that involves only cancer cells, in the last years different studies have braced the idea that different components surrounding the tumor are accountable for the tumor behaviour (150). According to that, the maintenance of the TME is an important step for the progression of the tumor, eventually leading to the acquisition of resistance against treatment. Cancer cells are able to grow and proliferate in this environment, not just hiding from the immune system thus preventing its eradication but using the surrounding system as an ally that guarantees its progression.

Components of TME are tissue resident cells and a large population of immune cells that are recruited. In fact, even in the first instance, the immune populations act as a cleaner in order to reduce cancer population(151). It is now known that the tumor develops escape mechanisms ensure that not only the tumor is able to escape from immune system but at the same time modify the TME in order to support the tumor and promote its progression. In this process, it is no coincidence that macrophages play a major role in promoting tumor progression towards increasing aggressiveness(152). Constituting up to 50% of a tumor mass, the macrophages with a tumor promoting role are normally classified as tumor associated macrophages TAMs (153), whose presence in the TME is always associated with poor prognosis in majority of the human cancers (154, 155).

The crosstalk between cancer cells and the components of TME happens through two principal pathways: or via a direct contact mechanism between cancer cells and another cell or with the extracellular matrix (ECM), or via the release of different soluble molecules such as cytokines, lipid mediators, and growth factors.

An important aspect that led the tumor to proliferate and grow in the tumor microenvironment is the maintenance in the TME of a chronic inflammation (156).

It is known that in homeostatic condition, the health of a tissue is primarily affected by leukocytes, that remove pathogens and foreign agents. when the homeostasis is altered by a potential treat the first response of the immune cells cause the release of cytokines and chemokines that activate and recruit other immune cell types, in order to activate an immune response against dangers (157, 158). Dendritic cells (DC) present in the tissue help the fight presenting the antigens thus activating the adaptive immune response. Once the threat is eliminated, inflammation resolves, and tissue homeostasis is re-stablished.

In tumor, on the contrary, this resolution never happens, thus generating a chronic inflammatory microenvironment(156). Cancer cells take benefit of this inflammation for two main reasons: firstly,

feeding continuously with mitogenic growth factors and cytokines, such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and fibroblast growth factors (FGF). Not to mention that in this environment the presence of proteolytic enzymes as (metallo, serine, and cysteine proteases) will help to modify the structure and functions of the tumoral ECM; these predominantly secreted by intratumoral macrophages, monocytes, and granulocytes (156, 159). In this scenario, cancer cells take advantage of the persistent presence of these factors, which instead of facilitating tissue repair, make cancer cells more aggressive(160).

Beside the tumor associated macrophages that play the most important role in the TME, other components contribute to it:

- The vasculature: one of the hallmarks of cancer is angiogenesis, they play a crucial role in tumor progression and metastasis (31). The vasculature that surrounds the tumor present altered structural and functional properties with areas of hypoxia and limited nutrient store. Hypoxia is an important aspect for maintenance of proliferation for cancer cells, in fact in this way it increases cell survival and resist to apoptosis induction. In this process the main actor is represented by the vascular endothelial growth factor VEGF, that is secreted predominantly by tumor cells (161).
- Cancer associated fibroblasts (CAFs): major component of the stromal cells, have been proved to provide critical signals that support progression and evade therapies (162). In normal circumstances, fibroblasts prevent the tissue integrity, *via* differentiation into myofibroblasts, which in turn facilitate the wound healing process and the repair of damaged tissue (163). The tumor itself exploits this function, with the help of the immune system that not only provides an uncontrolled proliferation of fibroblasts, but also stimulates them to release cytokines, chemokines, metabolites, enzymes, and ECM (164, 165). ECM induces cancer proliferation, migration and other processes supporting a malignant phenotype like angiogenesis and

epithelial to mesenchymal transition (EMT). When taking over this role, common fibroblasts turn “to the other side” to became cancer-associated fibroblasts (CAFs). Their indispensable work for the tumor is carried out at different levels: releasing chemokines such as CXCL2 and CCL7 (166, 167), or growth factors such as FGF and Hepatocyte growth factor (HGH) (168-170), as well as ECM proteins (171-173), which all favour the stages of tumor initiation and progression. The release of VEGF (174) instead, is important for the angiogenic processes, while the release of inflammatory molecules, such as IL-1β, IL-8 and TNFα (175, 176), allows the suppression of the immune system and provides the tumor an escape.

- Extracellular matrix: The ECM was initially considerate a stable structure whose only function was to provide structural support, nowadays is know that the ECM is a dynamic structure involved in various cellular processes, such proliferation migration and growth. Since it has been constituted by a network of structural protein as glycoproteins, and proteoglycans (177, 178), its main function is to maintain the architecture and to provide the nutritional support to the surrounding cells (179). The ECM also regulate the intracellular communication, favouring the passage of growth factors and cytokines, favouring intercellular communication. In cancer, the ECM is associated with the stromal tumor development by helping invasion progression and metastasis (179). Unlike the homeostatic condition, in the TME the ECM is unregulated and disorganized both from a physical and architectural point of view (180). Collage is the primary component of the ECM, that is fundamental for structural support, regulation of chemotaxis and cell migration. The amount of collagen in cancer is always doubled thus increasing the activity of the Matrix Metallopeptidase (MMP) and proteoglycans, thus facilitating the migration of cancer cells (181).

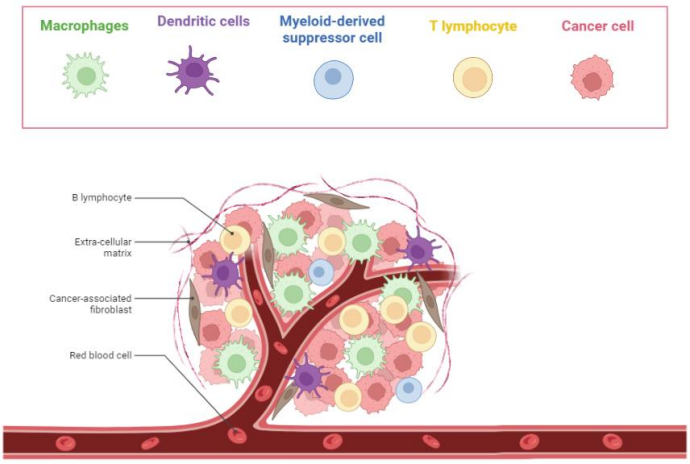

Figure 9 Schematic representation of Tumor microenvironment. Illustration adapted from "Tumor Microenvironment 2", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

3.1 Tumor Associated Macrophages (TAMs)

Macrophages play a pivotal role in the tumor microenvironment. The recruitment of macrophages at the tumor site promotes tumor growth, stimulates the tumor angiogenesis, and facilitates metastasis allowing the tumor cell to migrate and creating a favourable environment. All these phenomena cause a speed up tumor cell proliferation while reducing the efficacy of antitumor treatment. Since they are mainly responsible for the remodelling of TME, they can be considered a target for the development of new therapies. One of the main characteristics of macrophages is their ability to change their phenotype according to the stimuli that receive, showing a great plasticity and heterogeneity.

according to the common classification the macrophages can be categorized as M1 macrophages that play an antitumor part with inherent phagocytic and cytotoxic properties of macrophages in the early stages of tumor progression, and the M2 that facilitate tumor growth. Tumor associated macrophages display a M2 phenotypes being able to promote tumor growth and angiogenesis, as well as the capacity to remodel tissue and suppress the antitumor immunity (182).

The phenotypic switch operated by macrophages is none other than the result of a series of stimuli coming from the TME, and from the tumor cells themselves capable of releasing cytokines, guaranteeing to TAMs to sustain a continuous state of inflammation responsible for the pro-tumoral phenotype (183). Even if it is rare to encounter completely M1 or M2- polarized macrophages, the M1 macrophages are normally activated by INF- γ or cytokines like TNFα or IL-12, while the M2 phenotype is induced predominantly by IL-4, IL-6, IL-10 and CCL1 (184). All those cytokines are released from tumour occupying the TME and are able to induce those different phenotypes.

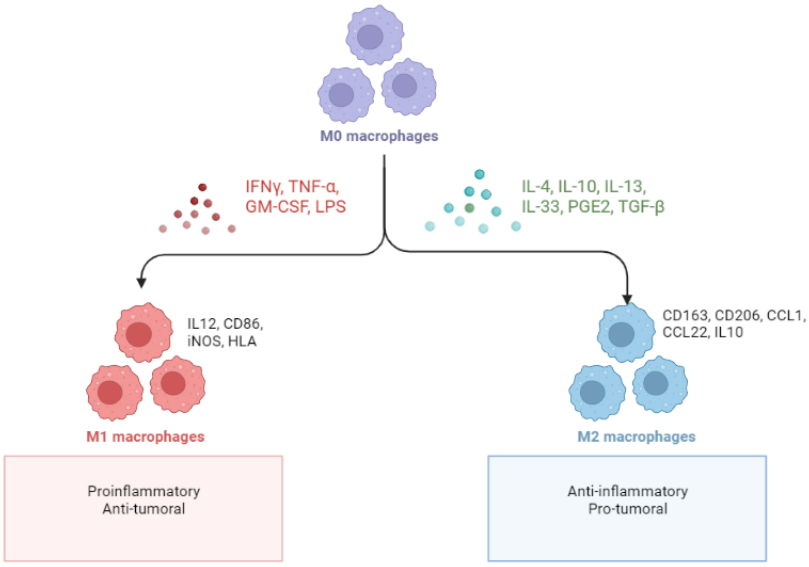

Figure 10 Macrophages polarization to M1 and M2 phenotypes . Illustration adapted from "Cell Differentiation Pathway (Layout, Vertical) 2" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

The number of macrophages as well as their phenotype change over the tumor progression. In fact, normally they increase in the early stages of tumor growth (185). The recruitment at the tumor site, is determined by tumor cells that secrete chemokine or other factors and induce a transduction signaling that recruits macrophages to the tumor site where they will be induced to polarize. Among the different factor, the colony stimulating factor 1 (CSF1) produced by tumor cells can facilitate

TAM aggregation and polarization (186). Once recruited macrophages start to produce a series of signaling molecules, as VEGF, IL-6, IL-10, reactive oxygen species (ROS) and TNFα in order to favour tumor growth (187). This interaction is clearly important to promote cell proliferation and metastasis, in fact, as the tumor progress, macrophages become an indicator of tumor malignancy and reflect cancer prognosis (182). The role played by macrophages in the ability to stimulate cancer progression has been proved in different kind of cancers, as gastric cancer (GC). GC cells interact with macrophages by secreting TNFα to generate CXCL1 and CXCL5, which trigger the CXCR2/STAT3 pathway to facilitate EMT and enable the migration of GC cells (188). GC mesenchymal stem cells (GC-MSCS) can accelerate metastasis by secreting IL-6 and CXCL8 to launch the JAK2/STAT3 signaling pathway. This event contributes to macrophage M2 polarization and stimulates EMT (189).The involvement of macrophages in tumor progression has been proved also in the initiation of metastasis. The main molecules produced in the TME that favour metastasis are the metalloproteinases (MMPs) because of their ability to degrade ECM. Among them, MMP2 and MMP9 can promote the production of new tumor blood vessels thus helping the process (190). Tumor cells then penetrate blood vessels and enter the bloodstream as circulating tumor cells (CTCs) (191). Proves of the necessary role played by macrophages in the regulation of metastasis has been apported by a study using zebrafish as model system to verify the interaction between tumor cells and macrophages. They found that M2 macrophages promote metastasis driving endosmosis thus suggesting a pivotal role of macrophages in the intravascular metastatic stage (192). TAMs are also involved in the formation of a premetastatic niche (193).

Another crucial step that requires the involvement of macrophages is angiogenesis. Nowadays there are different proves of the correlation between the degree of tumor angiogenesis with the number of TAMs in the tumor. In fact, via the secretion of vascular growth factors and MMPs macrophages promote the formation of new blood vessels (194, 195). Common proangiogenic factors include

VEGF, EGF, placental-derived growth factor (PlGF), platelet-derived growth factor (PDGF), TGF-α and TGF-β, and angiogenins 1 and 2. Among them, the top proangiogenic factors are PlGF and members of the VEGF family, i.e., VEGF-A VEGF-B, VEGF-C, and VEGF-D (196).

TAMs also interact with different kind of cells in the TME, including CAFs. It has been proved that the number of CAFs often correlates with the number of TAMs and the ability of TAMs to induce the transformation of stromal fibroblasts into CAFs (197). Different factors secreted by TAMs are strictly correlated with their activation as TGF-β or their migration via the MMP9 production (198, 199). In some kind of tumor like neuroblastoma, TAMs and CAFs are closely distributed thus suggesting an autocrine/direct interaction (200). In other cancers, the presence of CAFs correlates with the accelerated progression of tumor because of the presence of TAMs (201).

In the TME the second most abundant population of immune cells is represented by T cells. Based on the surface proteins and cytokines released, T cells, differentiate into separate subtypes with distinct immunological roles is possible. (202). Regulatory T cells (Tregs) are a subset of CD4+ T cells that promote tumor immune escape by suppressing antitumor immunity and contributing to tumor progression. Often a correlation between the numbers of Tregs and M2 macrophages, is associated with poor survival in patients with cancers (203). Tregs can promote the M2 phenotypes of TAMs via the inhibition of IFN-γ production by CD8+ T cells (204). Furthermore, TAMs produce CCL20 that facilitates Treg recruitment to the tumor (205), as well as silence the expression of STAT3 thus increasing the Treg production (206). In addition, in lung cancer, CCL22 produced by TAMs, stimulate the recruitment of Treg to the tumor site. Once Tregs are in the TME their production of CXCL8 stimulates TAMs to produce TGF-β (207). Treatment of cancer with inhibitors of Treg and TAMs has been proved to enhance the antitumor action (186). The interaction between TAMs and T

cells has been also proved with CD8+ T cells, which are known to have antitumor activity. Thus, TAMs are able to inhibit CD8+ T cells (208).

4. Animal Model for The Study of Cancer

4.1 Zebrafish as a model to study melanoma pathobiology

Together with mouse models, zebrafish represents a useful model to study cancer and, in particular, melanoma pathobiology. Like other vertebrates, zebrafish develops both benign and malignant cancers with histological, molecular, and pathological characteristics like human cancers (209). Although zebrafish rarely develops cancer spontaneously, tumours can originate in almost all tissues when the fish are exposed to water containing carcinogens (210).

The use of these fish as xenograft model has gathered praise in the scientific community for quality of results, primarily in the form of in vivo imaging in a whole vertebrate organism (64). The advantages that make zebrafish an attractive complementary model the fast development outside the mother, transparency at embryonic and larval stages, and the high number of progenies allowing for live imaging and cost-effective compound screening. Once the zebrafish genome was uncovered, has been revealed that zebrafish possess more of the 80% of all human disease-related genes, suggesting the possibility to model different human diseases. Focusing specifically on melanoma, the scientific community agree that the use of zebrafish for melanoma experiments is also determined by the simplicity of visualizing melanoma development. The use of tissue-specific promoters for the melanocyte lineage can help visualize melanocyte progenitors and differentiation in the living embryo and have been used to express oncogenes and tumor suppressor genes in these cells (Figure 3) (211). In addition, human melanoma cells injected into two-day-old zebrafish embryos were shown to survive, proliferate, migrate, and form tumor masses (Figure 4). Moreover, because of the

transparency of zebrafish embryos and their external development, studies can be carried out to evaluate the influence of the environment on melanoma cells and vice versa. A relevant advance for transplants was represented by the generation of adult zebrafish lacking body pigment cells, through spontaneous mutations of the *mitfa* and *mpv17* genes (212). The transparent fish that resulted, called Casper, allows clear visualization of the marked, transplanted cells. This gives scientists the chance to detect growth and metastasis of the pigmented melanoma cells. Both the embryo, which is transparent, and the adult fish provide remarkable resources for the characterization of melanoma cells. These tools are important in investigating the fundamental link between development and cancer and are a unique asset of the zebrafish system.

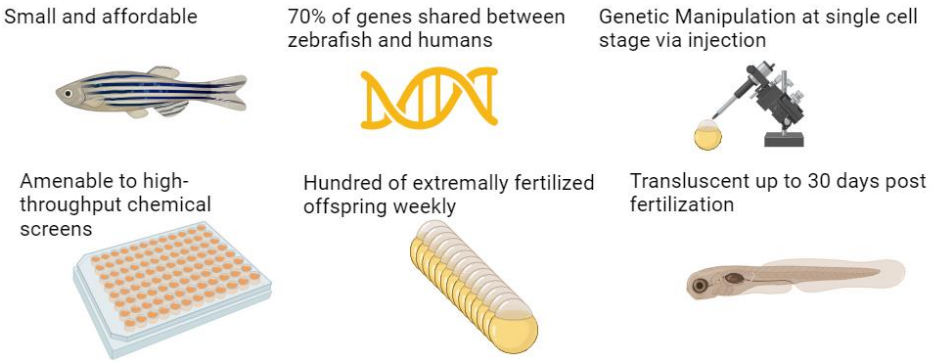

Figure 11 Advantages of the use of Zebrafish as a model System: The small dimensions that make zebrafish manageable, the effect that the 80% of the genome is shared with human, the possibility to be genetically manipulated at one cell stage, the possibility chemical screening, and its transparency at embryonic and larval stages make zebrafish an attractive complementary model. Created with BioRender.com

As masterfully explained by Langenau (213), zebrafish has become an important model system for the study of melanoma by providing insights and key knowledge in the basic biology of melanoma while offering new guidelines for the formulation of new therapeutic approaches (213). In fact, when used to analyse the interaction between tumor cells and tumor-associated macrophages, the xenografts of melanoma cells in zebrafish larvae provided key evidence of the strict interaction between melanoma cell lines and tumor associated macrophages, as shown by Roh-Johnson and collaborators (214). In this study the authors proved the motility of macrophages and the sustained contact with tumor cells, as well as the ability to promote cell dissemination when recruited. The relevance of zebrafish as a model system important for the study of melanoma was also provided by Lister et al. (215), whose study proved how when expressed at low level, MITF showed an oncogenic activity with BRAF(V600E) promoting melanoma progression. The study also evidenced that when removing MITF activity in BRAF(V600E) melanoma, a dramatic tumor regression marked by increased apoptosis was observed.

Several strategies were developed to study melanoma in zebrafish:

1. Xenotransplantation of human melanoma cells pre-stained with vital dyes (Figure 4).
2. Allotransplantation in adults, although xenotransplantation can also be possible after rearing of recipients at 37°C (216).
3. Visualization of early transformation using the *crestin:GFP* transgenic line (217). Cresting is expressed in neural crest during embryogenesis, but it is no longer expressed after 72 hpf. However, it is re-expressed in melanoma tumours when melanoma precursor cells re-initiate an embryonic neural crest signature.
4. The miniCoopR system has been developed by Dr. Li Zon and allows the rescue of melanin expression in Mitf-deficient fish, for example Casper (218). The miniCoopR method consists

of a plasmid that allows the gene of interest (GOI) to be placed under the *mitfa* promoter and also contains a *mitfa* minigene, allowing the easy tracking of transform cells because they recover melanin expression.

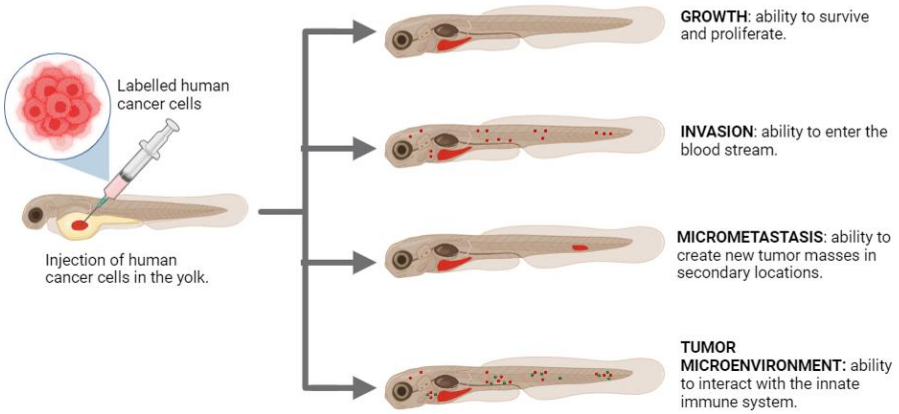

***Figure 12 zebrafish microinjection of cancer cells*:** Two dpf larvae are microinjected in yolk sack. After 3 dpi the effect of growth, invasion and micro-metastasis is analysed. Illustration adapted from "signs of cancer progression after zebrafish xenograft", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates. Retrieved from https://app.biorender.com/biorender-templates.

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4.2 Mouse as a Model to study melanoma pathobiology

The use of xenograft model since 1969 led to various studies that help understanding the cause, the prevention and therapy of various malignancies, including melanoma (219, 220) thus making the xenograft models a clinically relevant tumor model. Nowadays, human tumor xenograft models are used to test targeted therapies and to evaluate the effect of the combination of therapeutic agents (221, 222).

The mouse model represents the most widely preclinical model used in research, because of some of their characteristics as their easy manipulation and availability and the existing knowledge base regarding their genetics. Different kind of mouse model has been developed in the last years, including xenograft, genetically engineered and syngeneic models (223-225).

One of the most common ways is the xenotransplantation of human melanoma cell into immune-deficient mice. It is a useful mechanism to investigate key pathways that led to cancer cell development and progression. Immunocompromised mice are subcutaneously (SC) microinjected with human melanoma cells that cannot be rejected. The most common used models are nude athymic (nu/nu) mice that are T-cell deficient (226) or severe combined immune-deficient (SCID/SCID) mice that are deficient in both T-cells and B-cells (227).

Using this xenotransplantation melanoma cells can directly interact with the lymphatic and blood vessels, in order to evaluate the drug response and tumor behaviour in vivo (228). According to the process that is analysed different human melanoma cells lines can be used. Has been proved that different melanoma cell lines have the ability to metastasize spontaneously from primary xenografts to distant site, such as lung (229). An advanced mechanism of xenotransplantation requires the use of patient derived cells. The advantage of the patient-derived tumor xenografts (PDTXs) is represented by the possibility to maintain more similarities with the background of the patients, thus reflecting the cancer condition and preserving the fidelity of clinical characteristics (230).

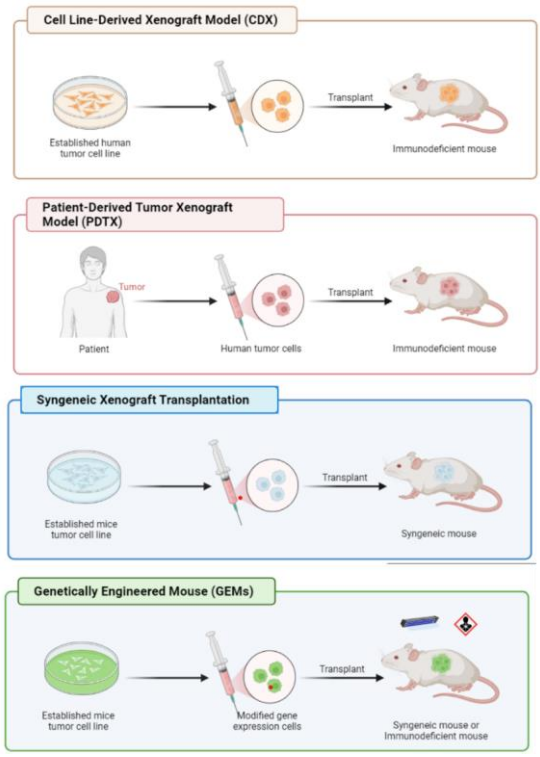


Figure 13 Schematic representation of mouse models used for the study of cancer. Illustration adapted from "Simplified Overview of Patient-Derived Xenograft Model (PDX)Patient-Derived Xenograft Model (PDX)", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

The syngeneic xenograft transplantation implies the induction of melanoma and the transplantation of melanoma cells into same species and genetic background. The xenograft model is often replace

with the Syngeneic xenograft transplantation that involves the induction and transplantation of melanoma cells into same species and genetic background (231). The choose of this kind of transplantation is associated with the possibility to evaluate the immune response once melanoma cells are transplanted and their interaction with the immune system. One of the most common cell lines used in the last years to study melanoma and metastasis is the B16 line that originated spontaneously for the first time in C57BL mouse model. The benefit in the use of this line is represented by the generation of two different subclones, obtained from in vivo passaging, the B16F1 and B16F10. While the B16F1 has a low metastatic potential thus making it useful for the study of the primary tumour growth, the B16F10 has high metastatic potential to distant visceral organs (232). The evident disadvantage of the mouse syngeneic transplantation model is the variability caused by differences between melanoma cells and the murine one.

The last model is represented by the Genetically Engineered Models (GEMs) that carry a modify gene expression helpful for the determination of tumorigenesis mechanisms. The importance of this model correlate with the possibility to elucidate the function of specific gene and to identify to therapeutic targets (233). In melanoma this model is often combined with other model for tumor induction, as carcinogens or ultraviolet to evaluate the predisposition and risk factors of melanoma development. Nevertheless, the side effects of the use of transgenic mouse models are many. Often, tumor can arise in different tissue limiting the use of the generated model, as well as the necessity for many mouse strains to be interbed in a costly and labor-intensive manner. The most common used melanoma engineered mouse model are:

1. CDKN2A models: Cyclin-dependent kinase inhibitor 2A (CDKN2A) locus was identified as a melanoma susceptibility region and encodes two well-identified tumor suppressor proteins, p16INK4A and p14ARF.

2. RAS models: RAS family of proteins was discovered to be frequently mutated in cutaneous melanoma (234) About 15%–25% of human melanomas harbour activating mutations in NRAS.
3. PTEN/BRAF models: Identification of the PTEN deletion and BRAF mutations were two of the greatest discoveries in melanoma field. BRAF carry somatic missense mutations in 65% of malignant melanomas (235). BRAF mutations are mutually exclusive to RAS mutations, and taken together, the mitogen-activated protein kinase (MAPK) signaling is overactivated in more than 85% of the malignant melanoma cases, as stated above.

OBJECTIVES

The general aim of this work is to study the non-canonical played by Bcl-xL on the crosstalk between melanoma cells and TAMs. The specific aims are:

1. Study the effects of melanoma-specific Bcl-xL on the recruitment and polarization of macrophages using mouse and zebrafish in vivo models.
2. Study in vitro the effect of melanoma-specific Bcl-xL on the migration and polarization of human monocyte-derived macrophages.
3. Identify the signaling pathways involved in the melanoma-specific Bcl-xL-mediated migration and polarization of macrophages.
4. Study the effects of the inhibition of the signaling pathways identified and TAM ablation on melanoma progression.

MATERIAL AND METHODS

1. Animal models

Zebrafish (*Danio rerio* H.) were obtained from the Zebrafish International Resource Center and mated, staged, raised and processed as described (24). The *Tg(mfap4.1:tomato)*^{xt12} (25), *Tg(tnfa:eGFP-F)*^{ump5} (26), *Tg(mpeg1:eGFP)*^{gl22} and *Tg(mpeg1:GAL4FF)*^{gl25} (27), and *Tg(UAS:NTR-mCherry)*^{c264} (28) were previously described. For in vivo invasion assay and macrophage polarization/recruitment, melanoma cells were labelled with 1,1′-di-octa-decyl-3,3,3′,3′-tetra-methyl-indo-carbo-cya-nine perchlorate (DiI, Molecular Probes, Invitrogen, Waltham, MA) and resuspended in a buffer containing 5% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, MA, USA) in PBS. Two hundred cells/embryo were then injected in the yolk sac of transgenic zebrafish larvae 2 dpf and maintained for 5 days at 35°C. Larvae were then analysed for melanoma cells dissemination and macrophages polarization and recruitment by fluorescence microscopy. Images acquisition was made using a Leica MZ16F fluorescence stereomicroscope and processed using Image J software.

The melanoma cell invasion score was calculated as the total number of melanoma cells present in the tail of larvae (126).

Mouse experiments with immunodeficient athymic CD1 nude mice were performed as previously reported (236).

2. Cell cultures and treatments

Human melanoma control (Mneo) and Bcl-xL overexpressing stable (Mxl90, Mxl7) cells derived from M14 cell line, A375 and monocytic THP-1 cells, were cultured in RPMI 1640 medium (Euroclone, Milan, Italy), supplemented with 10% inactivated FBS, 1% L-glutamine and 100 μg/mL penicillin/streptomycin (Euroclone). Mneo, Mxl90 and Mxl7 cells were obtained transfecting M14 line with a Bcl-xL expression vector (pcDNA3-Bcl-xL) and an empty vector (pc-DNA3) and cultured with G418 (800 μg/mL, Euroclone).

For siRNA transfection, cells were seeded and, after 24 hours, transfected with 20 nM pooled siRNA oligonucleotides against CCL5 (si-CCL5), control (si-control) or Bcl-xL (siBcl-xL) (siGENOME SMART pool, DharmaconRNA Technologies, Lafayette, Colorado, USA) by using INTERFERin (Polyplus, Illkirch, France) reagent. Transient transfection for the expression of mutated IKBα (IKBSR) protein was performed using JetPrime (Polyplus). Expression vectors encoding the human IκBSR was kindly provided by Cippitelli M.

For experiments with neutralizing antibodies, cells were treated with human anti-IL-1β (0.2 μg/mL), IL-8 (0.2 μg/mL) or CCL5 (0.2 μg/mL) (R&D Systems, Minneapolis, Minnesota, USA), antibodies for 24 hours.

3. Monocytes isolation and differentiation

Macrophages have been obtained from either THP-1 monocytes using 100 ng/mL phorbol-12-myristate- 13-acetate (PMA, Sigma-Aldrich, San Louis, USA) for 24 hours or healthy donor buffy coats, provided by Regina Elena National Cancer Institute, as previously reported. In particular, the peripheral blood mononuclear cells (PBMCs) were isolated using Lympholite-H (Euroclone) and plated in RPMI 1640 medium (Euroclone, Milan, Italy), supplemented with 10% inactivated FBS, 1% L-glutamine and 100 μg/mL penicillin/streptomycin. After 24h, monocytes were selected for their ability to adhere to the plate and were incubated for 10 days in RPMI 1640 supplemented with 10% of inactivated FBS and 50 ng/ml MCSF (Peprotech, London, UK) to obtain mature macrophages (monocyte-derived macrophages, M-DM). The, M-DM were stimulated for 24h in harvesting condition (serum free) and with CM derived from control or Bcl-xL overexpressing melanoma cells. In all experiments, the CM used for stimulating M-DM was normalized to the number of adherent cells. CM from M-DM was collected after 24h of stimulation with CM from melanoma cells, followed by further 24h in harvesting condition (serum free).

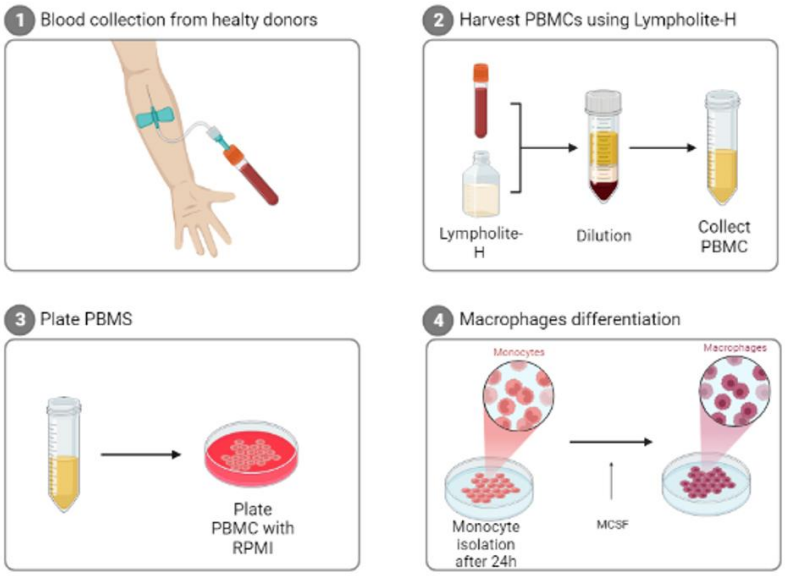


Figure 14 Monocyte isolation and differentiation Created with BioRender.com

4. Cell migration assay

Cell migration assay was performed using a chamber of Transwell (Costar, New York, USA) containing a 5 µm and 3 µm pore polycarbonate membrane for THP-1 and M-DM, respectively. One hundred thousand cells were plated in the upper chamber for 7 hours, with the CM derived melanoma cells in the lower chamber. The migrating cells were fixed and stained using the Differential Quick

Stain Kit (Dade Behring, Marburg, Germany) and photographed using light microscopy. The quantification was made by counting the migrated cells in 10 images for each condition.

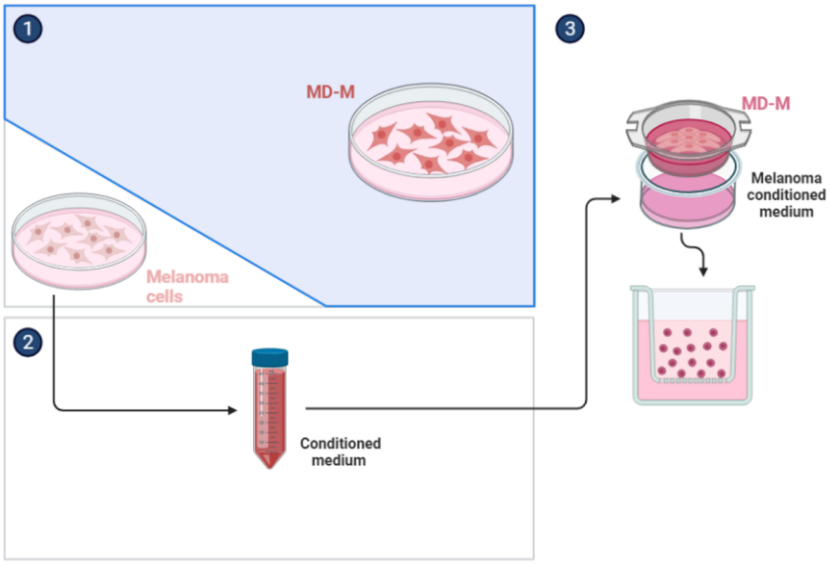


Figure 15 Cell migration assay using transwell: Created with BioRender.com

5. Elisa and Western blot analyses

ELISA assays for CXCL8, IL-1β (Enzo Life Sciences, New York, USA) and CCL5 (RayBiotech (Peachtree Corners, GA, USA) were performed using the CM from M14 melanoma cells, while ELISA for TGF-β (Fine Test, Whuan, China) was performed using CM from M-DM. Following manufacturer's instructions, each sample was evaluated in duplicate and protein levels were normalized to the number of adherent cells. Western blot was performed suspending the cellular pellet

with modified Ratio-Immunoprecipitation Assay (Tris-HCl 50 mmol/L pH 8, 150 mmol/L sodium chloride, 5 mmol/L EDTA, 0.1% sodium dodecyl sulfate, 1% NP-40, Sigma) placed on ice for 30 minutes, and centrifuged for 10 minutes at 4°C. The supernatants were collected and quantified using the BCA assay kit (Thermo Scientific, Rockford, Illinois, USA). Nuclear and cytoplasmic separation was performed by using the NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific) following the manufacturer's instructions. Proteins from cellular lysates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and subjected to immunoblot assays. Immunodetection was performed using antibodies against α-actin (Sigma-Aldrich), Bcl-xL, IKB, p65 and α-tubulin (Santa Cruz Biotechnology, Dallas, Texas, USA), HSP72/73 (Calbiochem, San Diego, California, USA), CCL5 (R&D Systems, Minneapolis, Minnesota, USA), Histone H3 (Cell signaling, Danvers, MA, USA). Anti-rabbit or anti-mouse IgG-horseradish peroxidase-conjugated antibodies (Amersham Biosciences, Freiburg, Germany) were used as secondary antibody. Densitometric analysis was performed using Image J software and normalized with relative α-actin or HSP72/73.

6. Human protein array

The Human Angiogenesis Antibody Array C1000 (RayBiotech. Inc., Peachtree Corners, GA, USA) was used according to the manufacturer's protocol to assess the secretion of more than 40 angiogenic factors into the CM of the different lines. Membranes spotted in duplicate with antibodies against angiogenic factors were blocked with blocking buffer and then were incubated overnight with CM. Next, membranes were washed with wash buffer, incubated with biotin-conjugated antibodies against proangiogenic factors, washed with wash buffer, and incubated with horseradish peroxidase–conjugated streptavidin. The signals on the membranes were detected by chemiluminescence. The

intensity of the protein signal (two spots for each protein) was compared with the relative positive signals by densitometric analysis.

Human Angiogenesis Antibody Array C1

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	ANG	EGF	ENA-78	bFGF
2	POS	POS	NEG	NEG	ANG	EGF	ENA-78	bFGF
3	GRO	IFN gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
4	GRO	IFN gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
5	PLGF	RANTES	TGF beta 1	TIMP-1	TIMP-2	THPO	VEGF	VEGF-D
6	PLGF	RANTES	TGF beta 1	TIMP-1	TIMP-2	THPO	VEGF	VEGF-D
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

Human Angiogenesis Antibody Array C2

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	ANGPT1	ANGPT2	PLG	Endostatin
2	POS	POS	NEG	NEG	ANGPT1	ANGPT2	PLG	Endostatin
3	G-CSF	GM-CSF	I-309	IL-10	IL-1 alpha	IL-1 beta	IL-2	IL-4
4	G-CSF	GM-CSF	I-309	IL-10	IL-1 alpha	IL-1 beta	IL-2	IL-4
5	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	TIE-2	TNF alpha
6	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	TIE-2	TNF alpha
7	uPAR	VEGF R2	VEGF R3	BLANK	BLANK	BLANK	BLANK	POS
8	uPAR	VEGF R2	VEGF R3	BLANK	BLANK	BLANK	BLANK	POS

Figure 16 Human Angiogenesis Antibody Array Map

7. RNA extraction and qRT -PCR

Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) was used to extract total RNA. Reverse transcription was performed using RevertAid Reverse Transcriptase (Thermo Scientific) kit and Gene-Amp 9700 PCR system (Applied Biosystems, Foster City, California, USA). qRT-PCR was performed using 7900HT Fast Real Time PCR system (Applied Biosystems), using the SYBR green dye detection method. The mRNA levels were normalized using α-actin. The list of primers for each gene was added to the Table 1. The results were evaluated by the 2-ΔΔCt method.

Comentado [VFMM6]: En la tesis no hay material suplementario. Tienes que poner la Table 1

Table 1 Primers used in RT-qPCR analysis.

GENE	FORWARD (5'-3')	REVERSE (5'-3')
CD163	CAGTCCCAAACACTGTCCTCGT	CAGGCGAAGTTGACCACTCTCTT
CD206	GCTGTTCTCCTACTGGACACCA'	AATCTGAGATTCGGACACCCA
CCL1	AAGAGCATGCAGGTACCCTTCT	CTCATTGGAGCAGATGGAGCT
CCL22	TGGCGTTCAAGCAACTGA	AAGRGTTTCACCACGCGCA
IL-10	GCGCTGTCATCGATTTCTTC	TGGCTTTGTAGATGCCTTTCTC
IL-12	GAGGCCTGTTTACCATTGGA	TCAAGGGAGGATTTTTGTGG
CD86	CACAGCAGAAGCAGCCAAAATG	TCTTCAGAGGAGCAGCACCAGA
HLA	GCCTCTTCTCAAGCACTGGGA	CCACCAGACCCACAGTCAGG
IL8	CACCGGAAGGAACCATCTCA	TGGCAAAACTGCACCTTCACA
IL1B	TGGCCCTAAACAGATGAAGTGC	CTGAAGCCCTTGCTGTAGTGGT
TNFA	GGAGGACGAACATCCAACCTT	CCCATTTCTCTTTTTGAGCCAG
MCSF	ATGCGCTTCAGAGATAACACCC	ATAGAAAGTTCGGACGCAGGC
CCL2	TCTCGCCTCCAGCATGAAAGT	GCATTGATTGCATCTGGCTGA
CCL5	TTTGTCACCCGAAAGAACCG	GAGGCAGAAACAGGCAAAT
ACTINA	ATTGCCGACAGGATGCAGAA	GCTGATCCACATCTGCTGGAA
IL1A	ATGAAGGTCGCATGGATCAATC	TCCCGTTGGCTACTACCAC
IL2	CAACTGGAGCATTTACTGCTGG	TCAGTTCTGTGGCCTTCTTGG
IL4	CCACGGACACAAGTGCGATAT	CTTGGAGGCAGCAAAGATGTCT
IL6	AAAGCAAAGAGGCACTGG	CCAGGCAAGTCTCCTCATTGAA

8. Immunohistochemical (IHC) analysis

IHC analysis of Bcl-xL expression and macrophage polarization or recruitment at the intratumoral or peritumoral area in melanoma xenografts from mice was performed as previously described (17), by using the following antibodies: anti-F4/80 (Thermo Fisher), anti-CD206 (Abcam, Cambridge, UK) or anti-Bcl-xL (Cell Signaling) antibodies.

9. Statistical Analysis

Statistical significance was determined by t test, a one-way ANOVA analysis of variance followed by a Newman–Keuls or Tukey post-test, Pearson's and Spearman's correlation coefficients using GraphPad Prism 7.04. For in vivo experiments the one-way Anova was made comparing the control of each group with the melanoma cells overexpressing Bcl-xL. The Mann-Whitney test was applied to compare the number of macrophages in the peritumoral or intratumoral area of melanoma tumours from immunodeficient mice.

RESULTS

1. Melanoma-specific Bcl-xL induces recruitment and polarization of macrophages in mouse and zebrafish models.

Starting from previous findings demonstrating that melanoma-specific Bcl-2 induces the recruitment and the polarization of macrophages to a M2 phenotype (236), we investigated the possible role exerted by melanoma-specific Bcl-xL in the regulation of macrophage status by using two different animal models, i.e., mouse and zebrafish. Thirty days after subcutaneous injection of control (Mneo) or Bcl-xL overexpressing (Mxl90) M14 melanoma cells in immunodeficient mice, the presence of macrophages infiltration was analysed by immunohistochemical (IHC) analysis using F4/80 and CD206 antibodies, the former being a specific murine macrophage related marker and the latter a specific M2 macrophage marker. As reported in Figure 13 A, B, a significantly higher number of both intratumoral and peritumoral M2 macrophage recruitment was observed in Bcl-XL overexpressing

xenografts when compared to control ones. IHC analysis also confirmed that Bcl-xL protein expression was maintained during in vivo growth (Figure 13A).

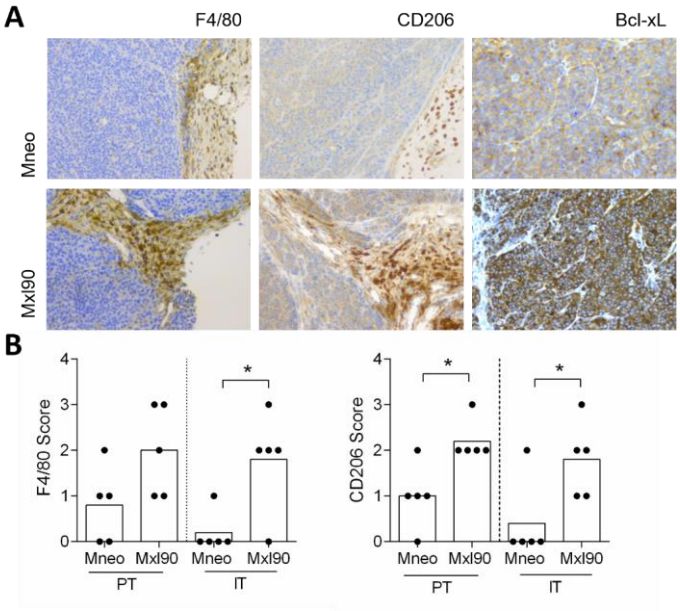


Figure 17) Melanoma-specific Bcl-xL induces recruitment and polarization of macrophages in mouse model A) Representative images of immunohistochemical analysis of F4/80, CD206 and Bcl-xL expression in melanoma control (Mneo) and Bcl-xL overexpressing (Mxl90) tumour-bearing mice, performed 30 days after subcutaneous cell injection (20X magnification), and B) relative quantification of peritumoral (PT) and intratumoral (IT) macrophage infiltration by F4/80 and CD206 staining. The results are reported as mean score: score 0, no detectable infiltrate; score 1, low infiltrate; score 2, moderate infiltrate; score 3, high or very high infiltrate. p- values were calculated between control and Bcl-xL overexpressing tumours. Each dot (•) indicates an experimental point, ***p<0.01;

To confirm these data in a different animal model, we took advantage of the use of xenotransplantation assay in zebrafish embryos (Figure 14). Specifically, parental and Bcl-xL overexpressing melanoma cells, were implanted in the yolk sack of 2 dpf zebrafish larvae from the transgenic line *Tg(mpeg1:mCherry; tnfa:eGFP)*, then macrophages cell polarization on the tail was analyzed 4 days post injection. This transgenic line shows macrophages in red (mCherry +) and Tnfa-expressing cells in green (eGFP +) allowing the visualization of M2 macrophages as mCherry + /eGFP - and M1 macrophages as mCherry + /eGFP +(237). As observed in mouse models (Figure 13A,B), a significant increased polarization of macrophages to a M2 phenotype was evidenced in larvae microinjected with melanoma cells overexpressing Bcl-xL when compared to larvae microinjected with parental cells (Figure 15A,B).

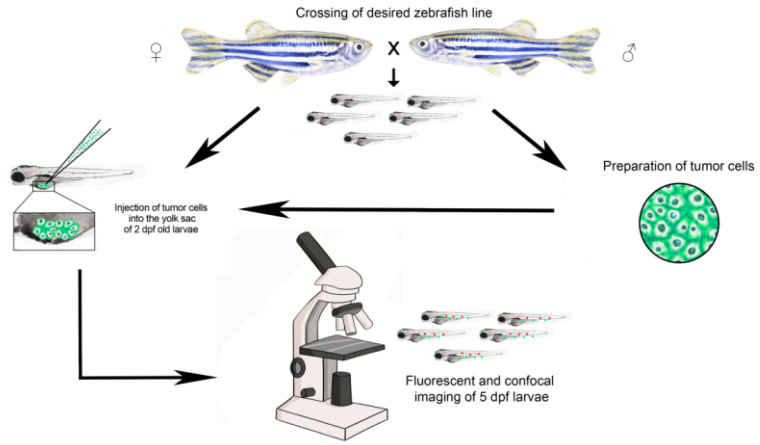


Figure 17 Schematic representation of melanoma cells microinjection in zebrafish larvae. Once obtained the larvae crossing zebrafish lines of interest, melanoma cells were prepared to be injected, marking them with a fluorescent stain.

At 2 days post fertilization (dpf), the larvae were injected into the yolk sac, and melanoma cell invasion was analysed at 5 dpf with fluorescent microscopy.

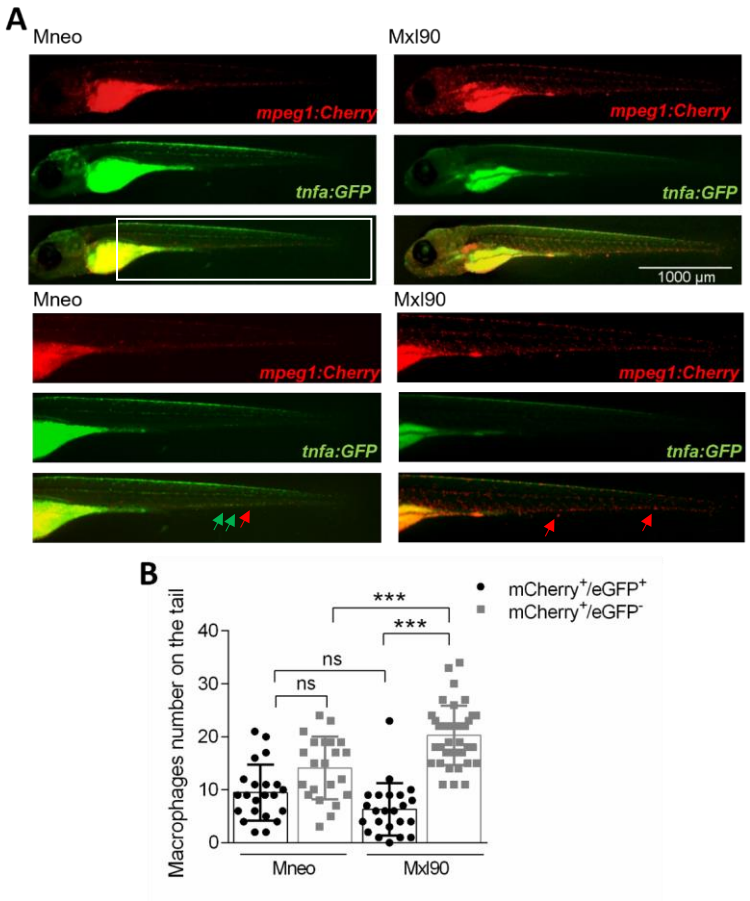


Figure 18 Melanoma-specific Bcl-xL induces recruitment and polarization of macrophages in zebrafish model A)Representative images of Mneo and Mxl90 cells 5 days after injection in the yolk sac of 2 days post fertilization

Tg(mpeg1: Cherry/tnfa:GFP) zebrafish larvae, and B) relative quantification of M1 (mCherry+/eGFP+; green arrows) and M2 (mCherry+/eGFP-; red arrows)) macrophages recruitment in the tail. The boxed area in the upper panels is magnified in the lower panels. p- values were calculated with One-way ANOVA analysis of variance test followed by Tukey's multiple comparisons test. B) Each dot (•) represent a larva, ***p<0.01; ns, no significant differences.

2. Melanoma-specific Bcl-xL induces in vitro polarization of human macrophages.

To deeply prove that Bcl-xL was able to induce the polarization of macrophages, human M-DM, obtained from healthy donor buffy coats, were stimulated with the culture medium (CM) from control (Mneo) or two different Bcl-xL overexpressing (Mxl90, Mxl7) melanoma cell lines. As reported in Figure 16A, a significantly higher expression of M2 markers (CD163, CD206, CCL1, CCL22 and ARG-1), paralleled by a significantly lower expression of M1 markers (IL-12, CD86, iNOS and HLA), was observed in M-DM exposed to CM from Bcl-xL overexpressing cells, when compared to CM from Mneo control cells. Similar results were obtained when THP-1 monocytes exposed to different CM (Figure 16B). Accordingly, M-DM stimulated with CM from Bcl-xL transfectants showed increase TGF-β secretion (Figure 16C). We also evaluated the M2-polarizing ability of CM from A375 (melanoma cell line with high levels of basal/endogenous Bcl-xL) silenced for siRNA control (A375 siCtrl) or for Bcl-xL (A375 siBcl-xL) (16D). As evident from the Figure 16E, CM

from A375 siBcl-xL partially blocked the M2 polarization induced by A375, causing a reduction of CD163, CD206, CCL1 and CCL22 M2 markers.

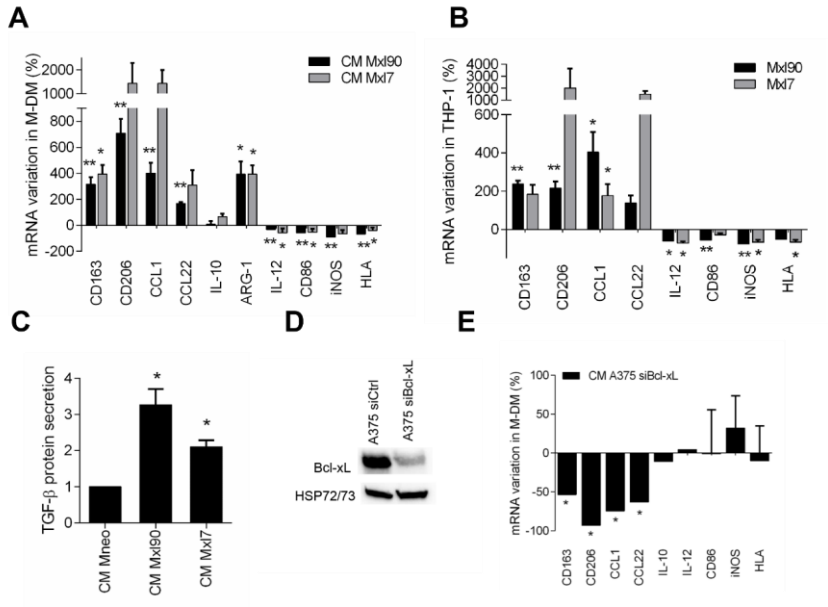


Figure 19. Melanoma-specific Bcl-xL induces in vitro polarization of human macrophages A) qRT-PCR analysis of CD163, CD206, CCL1, CCL22, IL-10, ARG-1, IL-12, CD86, iNOS and HLA levels in M-DM after 24 hours exposure to culture medium (CM) from control (Mneo) or Bcl-xL overexpressing (Mxl90, Mxl7) melanoma cells. The results are reported as percentage of mRNA variation in macrophages exposed to CM derived from Bcl-xL overexpressing cells versus control ones.B) qRT-PCR analysis of CD163, CD206, CCL1, CCL22, IL-12, CD86, iNOS and HLA levels in THP-1 cells after 24 hours exposure to culture medium (CM) from control (Mneo) or Bcl-xL overexpressing (Mxl90, Mxl7) melanoma cells derived from M14 cell line. A,B) p values were calculated between M-DM and THP-1 exposed to CM from Bcl-xL overexpressing cells and those exposed to CM from control cells. C) TGF-β protein secretion quantified

by ELISA essay in M-DM stimulated as reported in A). The results are reported as fold of TGF-β release in M-DM exposed to CM from Bcl-xL overexpressing cells versus control ones. D) Western Blot analysis of Bcl-xL levels in A375 cells silenced with siRNA control (siCtrl) or against Bcl-xL (siBcl-xL). HSP72/73 is shown as loading and transferring control. One representative western blot analysis out of two with similar results is reported. D) qRT-PCR analysis of CD163, CD206, CCL1, CCL22, IL-10, IL-12, CD86, iNOS and HLA levels in M-DM after 24 hours exposure to CM from A375 siCtrl or A375 siBcl-xL cells.

CM from melanoma cell of control and overexpressing Bc-xL were also used to evaluate their effect on M-DM migration. As reported in Figure 17A, a significant higher number of migrating cells was detected when M-DM cells were exposed to CM derived from Bcl-xL overexpressing cells and

compared to CM from control ones. Similar results were obtained when THP-1 monocytes were exposed to different CM (Figure 17B).

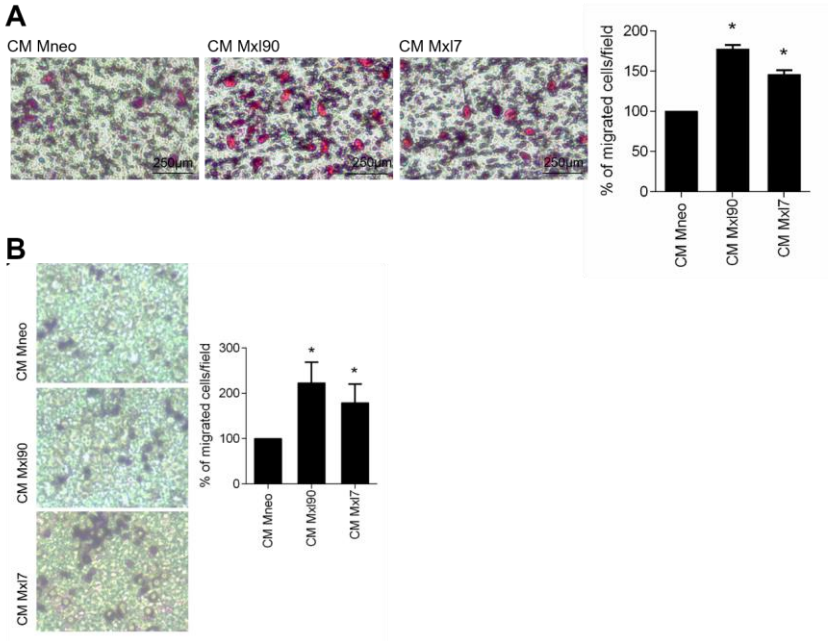


Figure 20. Melanoma-specific Bcl-xL induces in vitro recruitment of human macrophages A) Representative images (left panels) and relative quantification (right panel) of M-DM migration in response to CM from Mneo (CM Mneo), Mxl90 (CM Mxl90) or Mxl7 (CM Mxl7) cells. The quantification was performed by counting the number of migrated cells in at least 10 fields for each condition. The results are reported as percentage of migrated cells/field. p values were calculated between the percentage of M-DM migrated exposed to cells CM derived from Bcl-xL overexpressing cells, respect to the control melanoma cells B) Representative images (left panels) and relative quantification (right panel) of

THP-1 cell migration in response to CM from Mneo (CM Mneo), Mxl90 (CM Mxl90) or Mxl7 (CM Mxl7) cells. The quantification was performed by counting the number of migrated cells in at least 10 fields for each condition. The results are reported as percentage of migrated cells/field.

3. Melanoma-specific Bcl-xL exerts its effect on macrophage polarization and recruitment via the release of cytokines.

To investigate the possible mechanism underlying the process of macrophage polarization and migration induced by Bcl-xL, we performed a protein array comparing the CM derived from control and Bcl-xL overexpressing M14 cells. As reported in Figure 18A, the level of several molecules was found up- or down- modulated in the CM from Bcl-xL overexpressing cells, when compared to CM from control ones. Among the up-regulated molecules, we confirmed higher mRNA expression of

IL-8, IL-1β, IL-1α, TNFα, IL-2, IL-4 and IL-6, and the recruiting factor CCL5/RANTES by qRT PCR analysis (Figure 18B,C,D).

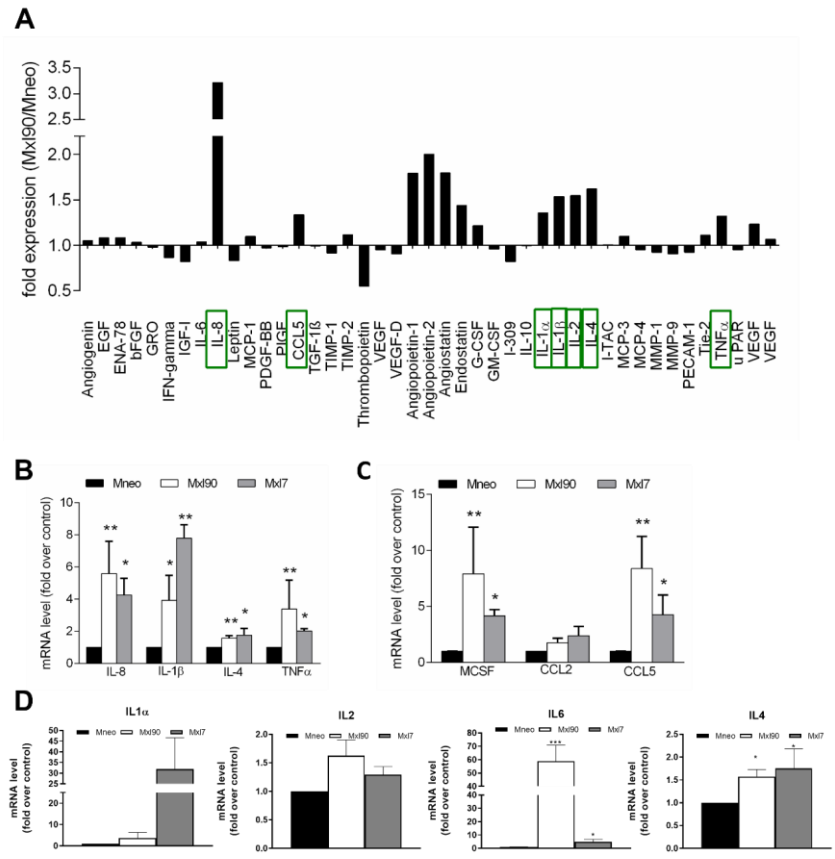


Figure 21. Melanoma-specific Bcl-xL exerts its effect on macrophage polarization and recruitment via the release of cytokines A) Quantification of the protein array probed with CM from control (Mneo) or Bcl-xL overexpressing (Mxl90) melanoma cells. Fold expression of Mxl90 relative to Mneo cells is reported. B,D) qRT-PCR analysis of B) IL-8, IL-1β, IL-4 and TNFα and C) MCSF, CCL2 and CCL5 in Mneo, Mxl90 and Mxl7 cells.D) IL-1a, IL-2 and IL6. B-F) The results represent the average±SEM of three independent experiments, p values were calculated between control and Bcl-xL overexpressing cells, *p<0.05, **p<0,01.

Higher levels of IL-8, IL-1β and CCL5 proteins were confirmed by western blot and ELISA analyses (Figure 19A,B,C). Among the other macrophage chemotactic factors beyond CCL5, macrophage-colony stimulating factor (MCSF/CSF1), not present in the protein array, has been also found up-regulated at the mRNA level after Bcl-xL forced expression, while the expression of the chemotactic factor CCL2/MCP-1 was not significantly modulated (Figure 3D).

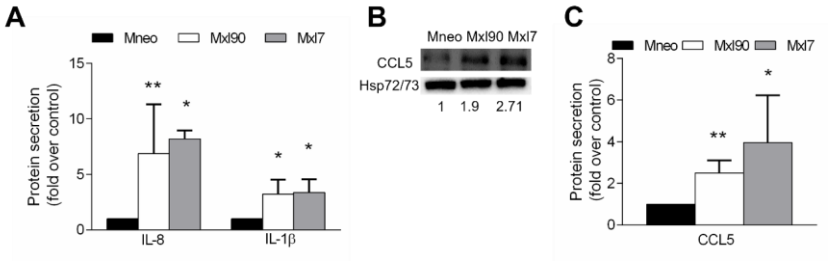


Figure 22 Analyisis of the protein level in Melanoma-specific Bcl-xL cells A) IL-8 and IL-1β protein secretion by ELISA in Mneo, Mxl90 and Mxl7 cells. B,C) CCL5 protein expression by E) Western blot analysis and F) ELISA in Mneo, Mxl90 and Mxl7 cells. HSP70 is shown as loading and transferring control. One representative western blot analysis out of two with similar results is reported. The numbers indicate densitometric analysis relative to control. The results represent the average±SEM of three independent experiments, p values were calculated between control and Bcl-xL overexpressing cells, *p<0.05, **p<0,01.

Using specific blocking antibodies, we next focused our attention on the role of IL-8 and IL-1β on macrophage polarization, and that of CCL5 on macrophage migration. As reported in Figure 20A,B, inhibition of IL-8 provoked a significant downregulation of the M2 markers CD163, CD206 and CCL1, while inhibition of IL-1β caused a significant downregulation of the M2 macrophage markers CD163 and CCL1, and a significant increase of the M1 macrophage markers CD86 and HLA. Importantly, M-DM (Figure 20C) and THP-1 (Figure 20D) cells showed a significantly reduced migration when exposed to CM from melanoma cells overexpressing Bcl-xL treated with CCL5

blocking antibody and compared to cells exposed to CM from Bcl-xL overexpressing cells treated with control siRNA.

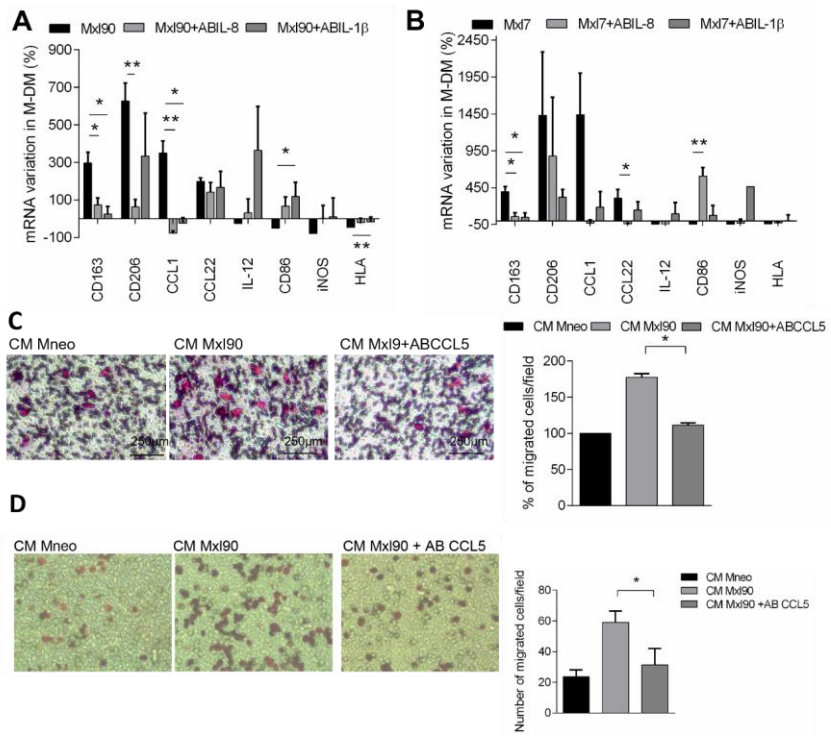


Figure 23 the use of blocking antibodies confirms the importance of these in the regulation of polarization and recruitment A) qRT-PCR analysis of CD163, CD206, CCL1, CCL22, IL-12, CD86, iNOS and HLA in M-DM stimulated for 24h with CM from Bcl-xL overexpressing A) Mxl90 and B) Mxl7 melanoma cells with or without anti-IL-1β (ABIL-1β) or anti-IL-8 (ABIL-8) blocking antibodies. Percentage of mRNA variation relative to untreated cells is reported. p values were calculated between Bcl-xL overexpressing cells untreated and treated with antibody. C) Representative

images (left panels) and relative quantification (right panel) of M-DM migration in response to CM from control (CM Mneo), Mxl90 untreated (CM Mxl90) or treated with the blocking antibody CCL5 (CM Mxl90+ ABCCL5). The quantification was performed by counting the number of migrated cells in at least 10 fields for each condition. p values were calculated between M-DM migrated cells exposed to CM derived from Bcl-xL overexpressing clones treated and untreated with antibody. D) Representative images and relative quantification of THP1 cell migration in response to culture medium (CM) from Mneo (CM Mneo), Mxl90 (CM Mxl90) untreated or treated with anti-CCL5 antibody (CM Mxl90+ABCCL5). The quantification was performed by counting the number of migrated cells in at least 10 fields for each condition.

We next evaluated the effect of CCL5 silencing by siRNA on the ability of Bcl-xL overexpressing melanoma cells to recruit macrophages after microinjection of melanoma cells in the zebrafish line *Tg(mfap4:tomato)*, which has red labelled macrophages. As reported in Figure 21B-E, while Bcl-xL overexpressing melanoma cells silenced for CCL5 do not show less ability to invade the tail,

inhibition of CCL5 significantly reduced the capacity of macrophages to be recruited at the tumor site, resulting in reduced interactions between invading tumor cells and macrophages.

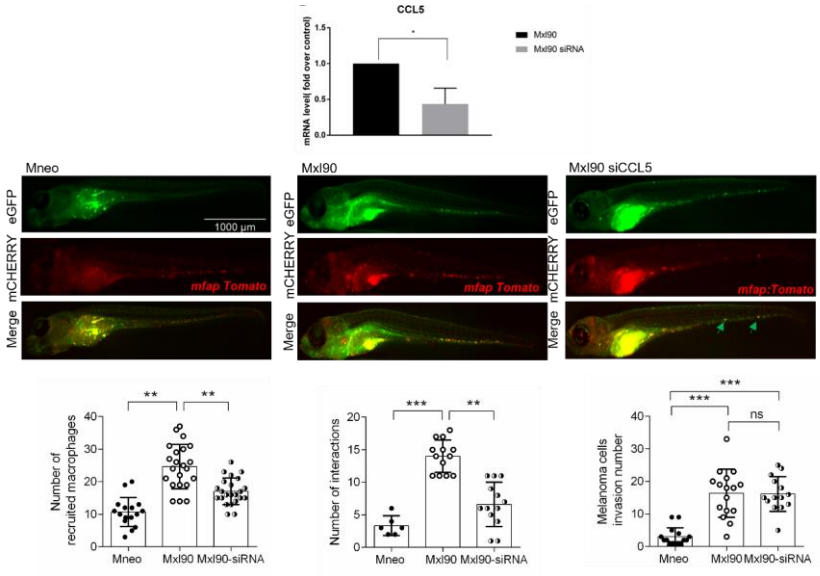


Figure 24 CCL5 regulates macrophage recruitment but not their expression A) qPCR of CCL5 expression in Mxl90 cells after the silencing with siRNA B) Representative images of Mneo, Mxl90 and Mxl90 cells silenced for CCL5 (Mxl90 siCCL5) stained in green 5 days after injection in the yolk sac of 2 days post fertilization *Tg(mfap4:Tomato)* zebrafish larvae, and relative quantification of C) recruited macrophages, D) interaction between melanoma cells and macrophages counted as yellow spots (green arrows), and E) invasion score of melanoma cells, p values were calculated with one-way ANOVA analysis of variance test followed by Tukey's between C, E) number of melanoma cells and macrophages recruited in the tail of zebrafish larvae silenced or not for CCL5, D) number of interaction (yellow spot) between macrophages and melanoma cells silenced or not for CCL5 via one-way ANOVA analysis of variance test followed by Tukey's multiple comparisons test, *p<0.05, **p<0,01. ***p<0,001.

4. Melanoma-specific Bcl-xL regulates IL-8, IL-1β, MCSF and CCL5 expression through NF-κB

As up-regulated genes (IL-8, IL-1β, MCSF and CCL5) in Bcl-xL overexpressing melanoma cells have in common that are all regulated by NF-κB (126, 238), we evaluated if this transcription factor was the intermediary between Bcl-xL and the release of these cytokines. To this purpose, we blocked the nuclear translocation of p65 transfecting Bcl-xL overexpressing melanoma cells with IKBSR (Figure 22 A,B), the mutated form of IKB, that acts as a NF-κB super repressor (239) , and tested the effect on macrophage polarization and migration. We demonstrated that the inhibition of NF-κB was able to reduce IL-8, IL-1β and CCL5 expression at both mRNA (Figure 22A) and protein (Figure 22B) levels. We also evidenced a significantly reduced ability of M-DM (Figure 22C) and THP-1

(Figure 22D) cells to migrate in response to CM from Bcl-xL overexpressing cells transfected with IKBSR compared to cells exposed to CM from control transfected Bcl-xL overexpressing cells.

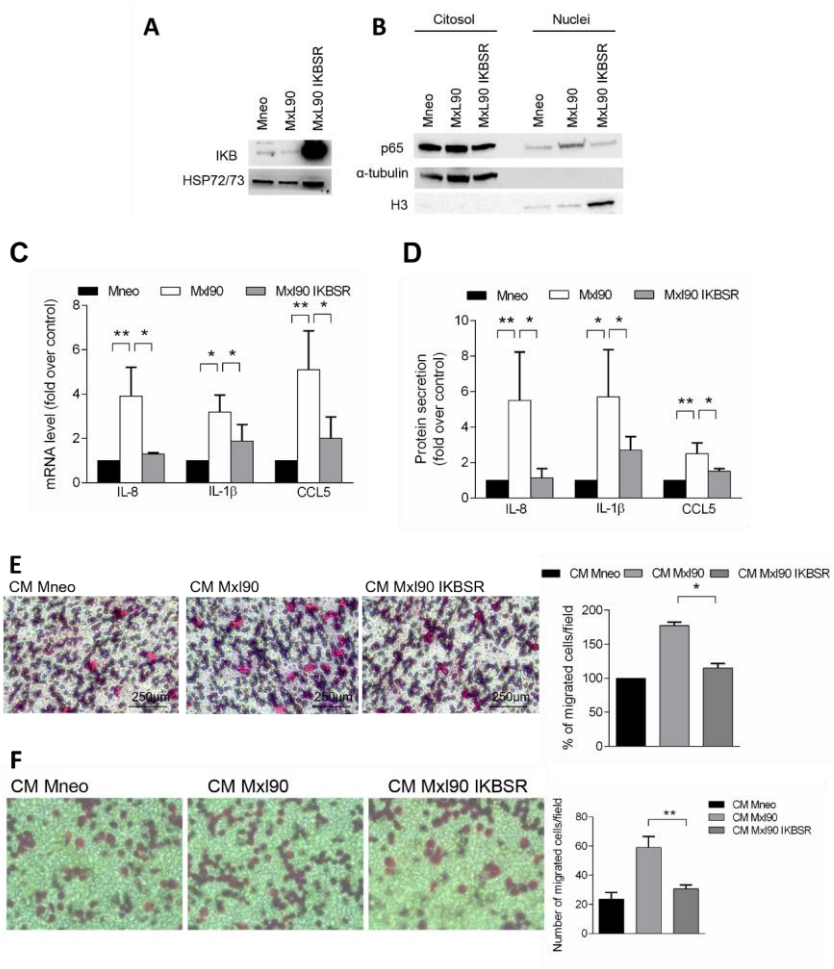

A
Mneo
MxL90
MxL90 IKBSR
IKB
HSP72/73
B
Citosol
Nuclei
Mneo
MxL90
MxL90 IKBSR
Mneo
MxL90
MxL90 IKBSR
p65
α-tubulin
H3
C
Mneo
Mxl90
Mxl90 IKBSR
mRNA level (fold over control)
IL-8
IL-1β
CCL5
D
Mneo
Mxl90
Mxl90 IKBSR
Protein secretion (fold over control)
IL-8
IL-1β
CCL5
E
CM Mneo
CM Mxl90
CM Mxl90 IKBSR
250um
250um
250um
CM Mneo
CM Mxl90
CM Mxl90 IKBSR
% of migrated cells/field
F
CM Mneo
CM Mxl90
CM Mxl90 IKBSR
CM Mneo
CM Mxl90
CM Mxl90 IKBSR
Number of migrated cells/field

Figure 25 Melanoma-specific Bcl-xL regulates IL-8, IL-1β, MCSF and CCL5 expression through NF-kB A,B) Western Blot analysis of A) IKBα in the total extract and B) p65 in nuclear and cytosol extracts in control (Mneo) or Bcl-xL overexpressing (Mxl90) melanoma cells derived from M14 cell line, and in Mxl90 IKBSR cells. A) HSP72/73 is shown as loading and transferring control. B) α-tubulin and histone H3 are shown as loading, transferring and cytoplasmic/nuclear purification control. One representative western blot analysis out of three with similar results is reported. C) qRT-PCR analysis and D) ELISA of IL-1β, IL-8 and CCL5 expression in control (Mneo) and Bcl-xL overexpressing melanoma cells transfected with IKBSR (Mxl90 IKBSR) or control (Mxl90) vectors. Fold induction relative to Mneo cells and the average±SEM of three experiments is reported. The average±SEM of three independent experiments is reported. E) Representative images (left panels) and relative quantification (right panel) of M-DM migration in response to CM from Mneo (CM Mneo), Mxl90 (CM Mxl90) and Mxl90 IKBSR (CM Mxl90 IKBSR) cells. The quantification was performed by counting the number of migrated cells in at least 10 fields for each condition. t-test analysis was used to calculate the p values between the percentage of migrated cells exposed to CM derived from Bcl-xL overexpressing clone transfected with CMV or IKBSR. F) Representative images and relative quantification of THP1 cell migration in response to CM from Mneo, Mxl90 and Mxl90 IKBSR cells. The quantification was performed by counting the number of migrated cells in at least 10 fields for each condition. **p<0.01.

We next evaluated the effect of NF-κB inhibition on the ability of Bcl-xL overexpressing melanoma cells to invade the caudal region after microinjection in zebrafish larvae of 2 dpf from the transgenic line with green-labelled macrophages *mpeg1:eGFP*. As reported in Figure 27, inhibition of NF-κB was able to reduce Mxl90 melanoma cell invasiveness compared to control Mxl90 cells, with a rate of invasion like that of parental Mneo melanoma cells. Strikingly, inhibition of NF-κB in Bcl-xL

overexpressing melanoma cells also reduced the number of recruited macrophages, phenocopying the effects of CCL5 inhibition (Figure 5D,E).

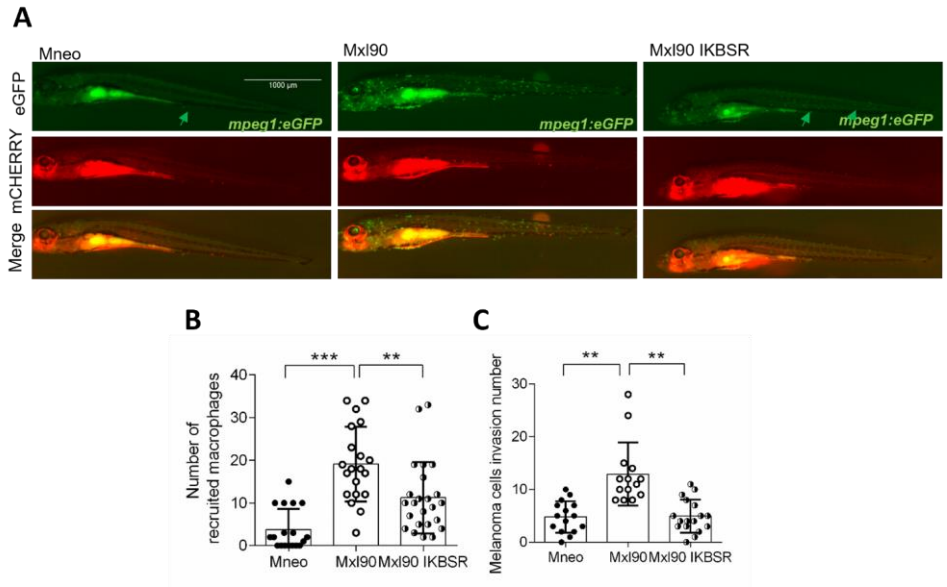


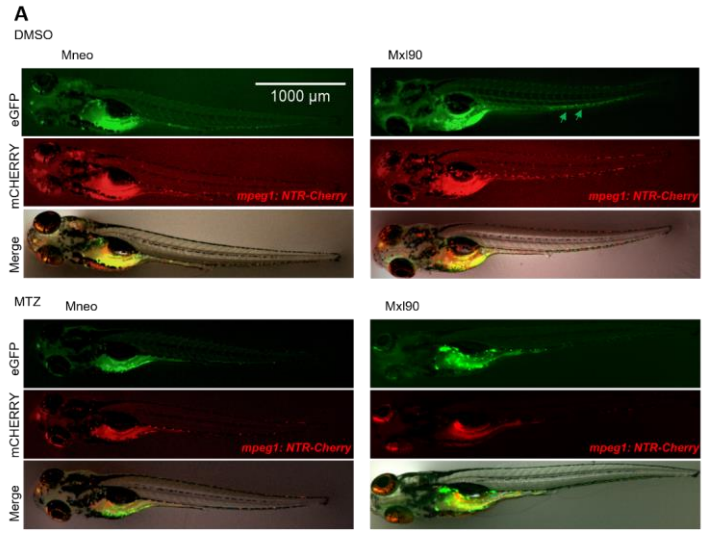
Figure 26 effects of NF-κB inhibition on the ability of Bcl-xL overexpressing melanoma cells to invade A) Representative images of red-stained Mneo, Mxl90 and Mxl90 IKBSR cells 5 days after injection in the yolk sac of 2 days post fertilization *Tg(mpeg1:EGFP)* zebrafish larvae, and relative quantification of recruited macrophages (B) and invasion score of melanoma cells invading the tail (C). p values were calculated with one-way ANOVA analysis of variance test followed by Tukey's between. *p<0.05, **p<0,01, ***p<0,001

Comentado [VFMM7]: Falta Merge

5. Depletion of macrophages causes a reduced capacity of melanoma cells to migrate

To explore the effect of macrophages ablation on melanoma invasion in the zebrafish xenograft model, we took advantage of the *Tg(mpeg1:GAL4FF, UAS:NTR-mCherry)* line that expresses bacterial nitroreductase (NTR) fused to mCherry in macrophages, allowing the conversion of the prodrug metronidazole (MTZ) in a cytotoxic compound that causes macrophage death (240). As reported in Figure 28, macrophage depletion reduced the invasiveness of Bcl-xL overexpressing

melanoma cells to the levels observed in Mneo parental cells. As expected, MTZ has no effect on the migration of melanoma cells in wild type larvae that did not express NTR (data not shown).



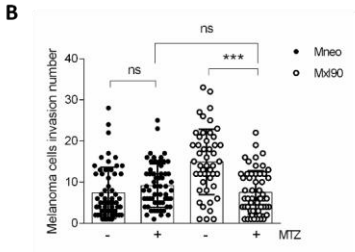


Figure 27 Depletion of macrophages causes a reduced capacity of melanoma cells to migrate *A*) Representative images and B) relative quantification of invasion score of green-stained control (Mneo) and Bcl-xL overexpressing (Mxl90) melanoma cells 3 days after injection in the yolk sac of 2 dpf larvae of the *Tg(mpeg1:GAL4FF, UAS:NTR-mCherry)* line expressing bacterial nitroreductase (NTR) fused to mCherry in macrophages, treated with DMSO or 10 mM Metronidazole (MTZ). Green arrows show invading melanoma cells. p values were calculated with One-way ANOVA analysis of variance test followed by Tukey's, ***p<0,001; ns, non-significant.

DISCUSSION

Melanoma represents the deadliest form of skin cancer. Features of melanoma are the high aggressiveness and the poor responsiveness to standard therapeutics. Despite the great advances that have been made in the last years thanks to the advent of both targeted therapies, designed against factors of the BRAF/MEK pathways, and immunotherapy-blocking immune checkpoints, significant safety issues remain in relation to the activation of a toxic response in the immune system of the host and in the emergence of drug resistance. Several clinical trials are ongoing to overcome these limitations and to evaluate in unresectable or advanced melanoma the effect of immune checkpoint inhibitors in combinatorial regimes with targeted therapy (NCT01767454), other immune modulators such as interleukin-2 (ClinicalTrials.gov Identifier: NCT0456212) and IDO (ClinicalTrials. gov Identifier: NCT02073123), therapeutic cancer vaccine (ClinicalTrials.gov Identifiers: NCT04382664, NCT04697576) or stereotactic body radiation therapy (ClinicalTrials.gov Identifier: NCT03693014). Molecular mechanisms leading to melanoma development, progression and response to therapy are the focus of intense investigation, aimed at understanding its pathobiology, the molecular mechanism of drug resistance and, finally, at developing new treatment strategies. Understanding the factors that promote progression of melanoma could help physicians to predict its probable course, thereupon planning treatment and anticipating further care.

TME has acquired in the last years an important role in the regulation of tumor behaviour. Macrophages, which are often closely related to poor prognosis, play a critical role in TME, exerting strong tumor-modulating effects by way of switching their phenotypes (153-155, 241, 242). Among factors able to recruit and polarize macrophages, we previously identified melanoma-specific Bcl-2 protein (236). The goal of this study was to evaluate whether the ability to affect macrophage

functions was restricted to Bcl-2 or generalized to other anti-apoptotic proteins such as Bcl-xL, and to investigate the molecular mechanism of Bcl-xL-driven macrophages switch. Previous studies from our and other groups evidenced multifaceted roles of Bcl-xL, acting not only as an anti- apoptotic factor, but also being involved in processes strictly related to tumor progression, such as angiogenesis, stemness and EMT (20, 71, 126, 236). In addition, the relevance of Bcl-xL in cancer patients and in particular, in those affected by melanoma, has been well established. Bcl-xL is expressed at lower levels in nevi, while its expression is increased in advanced melanoma compared with primary ones (20). By using in vitro melanoma and monocyte cells and two different in vivo models, i.e., mouse and zebrafish, in this study we demonstrated that melanoma-specific Bcl-xL promotes the recruitment of macrophages at the tumor site and induces a pro-tumoral M2 phenotype, though the release of soluble factors. Even if Bcl-2 and Bcl-xL affect macrophages function via common factors, from our studies differences emerged between the two anti-apoptotic proteins. In particular, the effect of specific neutralizing antibodies on macrophages functions evidenced that while IL-1β plays a relevant role in both Bcl-2- and Bcl-xL-induced macrophage differentiation, CCL2 and CCL5 were evidenced as chemokines involved, respectively, in Bcl-2 and Bcl-xL ability to induce macrophage recruitment. Our study also demonstrates that Bcl-xL induced expression of IL-8 mediates the effect of Bcl-xL on macrophage polarization. It also indicates that melanoma-specific Bcl-xL could also affect macrophage status through the induction of IL-4 and MCSF. As crosstalk between MCSF and IL-8 has been reported in terms of reciprocal induction in different model systems (243, 244), we can also hypothesize an indirect effect of Bcl-xL on these two factors. Similarly, Bcl-xL induction of MCSF could be indirectly related to TNFα induction previously evidenced by other authors (245). As we also demonstrated that melanoma specific Bcl-xL induced the expression of Angiopoietins 1 and 2, the ligands of Tie-2 receptor, we cannot exclude that other cytokines, positively regulated by Bcl-xL such as angiopoietin-2, could play a role in macrophage functions, through their ability to enhance

tumor-infiltration and proangiogenic or immunosuppressive activities of TIE2-expressing macrophages (246, 247). Further experiments are needed to validate this hypothesis and to understand the relevance of the angiopoietin 2/Tie-2 pathway in our experimental model.

Several evidences support the relevance in melanoma progression and response to therapy of chemokines induced by Bcl-xL in this study: we previously reported Bcl-xL-mediated secretion of IL-8 with consequent promotion of aggressiveness (123, 126); aberrant expression of IL-1β promotes inflammation, invasion, migration and growth as well as the stemness (248-250); CCL5 is secreted by melanoma cells and is related to tumorigenesis and progression, and affects tumor immune responses (251-253); and inhibition of MCSF shows anti-tumor efficacy (254, 255). Our findings are in agreement with those demonstrating i) IL-1β ability to regulate macrophage functions (256, 257), ii) the ability of IL-8 neutralization to attenuate the promoting effect induced by GC-MSCs on M2-like macrophage polarization (189), and iii) correlation of CCL5 expressions with infiltration of macrophages in cutaneous melanoma (258). Once in contact with these cytokines, macrophages undergo a phenotypic and functional switch promoting an anti-inflammatory behaviour that regulates malignant progression, through increased angiogenesis and tumor cell invasion (152, 153, 241).

As IL-8 (259), IL-1β (260) and CCL5 (261) are all regulated by NF-κB, we also evaluated whether NF- κB was the common transcription factor through which Bcl-xL induced these chemokines in our models: through genetic approaches inhibiting NF-κB functions we found that all these chemokines are regulated by Bcl-xL in a NF-κB dependent manner. Moreover, interfering with NF-κB activity reduced in vitro and in vivo migration of both macrophages and melanoma cells. In contrast, inhibition of CCL5 production by melanoma cells only affected the recruitment of macrophages, suggesting that primary tumor-derived factors can impact the function of resident macrophages in distant sites to sustain tumor aggressiveness. Whatever the outcome, our findings are in agreement

with evidences demonstrating the importance of NF-κB in melanoma progression (262, 263), and the relevance of Bcl-2 and Bcl-xL to regulate cancer progression-associated properties through NF-κB. In particular, we previously reported that Bcl-xL and Bcl-2 increased the NF-κB DNA binding activity with a mechanism dependent to IKKα/β and IKBα phosphorylation in glioblastoma and breast cancer (71, 126, 264). Our data strongly suggest that the previously reported tumor/resistance promoting effect exerted by Bcl-xL could also be related to the induction of different chemokines. We can hypothesize that, in addition to their effect on macrophages phenotype, Bcl-xL-induced cytokines could also promote a crosstalk between tumor cells and other stromal cells requiring these cytokines for their function. In particular, Bcl-xL-induced regulation of chemokines could affect the ability of i) CCL5 to drive NK cell infiltration in melanoma (265) or the response to immunotherapy (252, 266) ; ii) MCSF to induce a BRAF inhibitor resistant phenotype (267) , or to promote fibroblasts activation or myeloid derived suppressor cells migration (268); iii) TNFα to contribute to melanoma invasion/growth and tolerance to MAPK inhibition (269, 270) and to MCSF secretion in macrophages (245), as well as to cooperate with IL-4 in protecting cancer cells from apoptosis (271). Therefore, our study paves the way for future investigations aimed to decipher the role of melanoma-specific

Bcl-xL on the manipulation of macrophage behaviour to increase tumor aggressiveness and highlight the usefulness of combining different animal models.

CONCLUSIONS

The results obtained with this work lead to the following conclusions:

1) The overexpression of Bcl-xL in melanoma cells promotes macrophage recruitment to the tumor and M2 polarization in both mouse and zebrafish models.
2) Human monocyte-derived macrophages and monocytic THP-1 cells are polarized to a M2 phenotype when exposed to the culture medium of melanoma cells overexpressing Bcl-xL. In addition, the culture medium of melanoma cells overexpressing Bcl-xL also promotes human monocyte-derived macrophage migration.
3) The culture medium from melanoma cells overexpressing Bcl-xL induces in vitro M2 polarization and migration of human monocyte-derived macrophages and monocytic THP-1 cells.
4) Melanoma cells overexpressing Bcl-xL exerts its effect on macrophage recruitment and polarization through the release of cytokines. While IL-8 and IL-1β are involved in the polarization of macrophages, the chemokine CCL5/RANTES mediates macrophage recruitment to the tumor.
5) The regulation of cytokines by Bcl-xL in melanoma cells is dependent of NF-κB. Thus, genetic inactivation of NF-κB results in reduced cytokine release and macrophage migration in human and zebrafish melanoma models.
6) The depletion of macrophages in zebrafish xenograft models results in reduced invasiveness of Bcl-xL overexpressing melanoma cells.

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RESUMEN EN CASTELLANO

Introducción

El melanoma es la forma de cáncer de piel más peligrosa y mortal y representa más del 75% de todas las muertes por cáncer de piel. A pesar de los nuevos avances en el tratamiento del melanoma, el pronóstico sigue siendo malo, con una tasa de supervivencia a cinco años del 16 % para pacientes con metástasis a distancia, y su incidencia sigue aumentando. El melanoma se origina en los melanocitos, las células productoras de pigmento presentes en la capa basal de la epidermis, generalmente cuando el daño del ADN no reparado desencadena mutaciones que hacen que las células se multipliquen rápidamente y formen tumores malignos. El melanoma es causado principalmente por la exposición intensa a la radiación ultravioleta. Otros factores, como un sistema inmunitario comprometido o factores de riesgo congénitos como la cantidad de lunares benignos, el tipo de piel o los antecedentes familiares, también afectan a la aparición del melanoma. La progresión y el desarrollo del melanoma se caracteriza por dos etapas de crecimiento: radial y vertical. Durante la etapa de crecimiento radial, las células malignas crecen en un movimiento hacia afuera, extendiéndose por la epidermis. Con el tiempo, la mayoría de los melanomas progresan a la etapa de crecimiento vertical, durante la cual las células malignas invaden la dermis y desarrollan la capacidad de diseminarse o hacer metástasis. Una de las principales rutas afectada en el melanoma es la vía RAS-RAF-MAPK/ERK, cuya activación constitutiva a menudo se asocia con el desarrollo de melanoma. Esta vía controla la proliferación celular, la invasión, la angiogénesis y la metástasis. Otro factor comúnmente asociado al melanoma es el factor de transcripción asociado a la microftalmía (MITF). Es un factor de trascripción maestro que regula la proliferación y diferenciación de los melanocitos.

Unas de las características del cancer es una apoptosis alterada, que no permite mantener una homeostasis continua de los tejidos. Las vías apoptóticas tienen como base del proceso la activación de caspasas, que degradan componentes celulares para ser reconocidos por los fagocitos. Se conocen hasta la fecha dos vías diferentes en la activación de este proceso, de las cuales una se define comúnmente como "intrínseca" y mediada por mitocondrias, y la otra como "extrínseca" y mediada por receptores de muerte. Los reguladores clave de este proceso son miembros de la familia del linfoma de células B 2 (Bcl-2) que, después de la activación, causan primero una permeabilización de la membrana externa mitocondrial y luego una liberación de citocromo C de la mitocondria al citosol, determinando la activación de la cascada de caspasas a través de la caspasa 9. Los miembros de la familia Bcl-2 se clasifican según su estructura y función. Los miembros anti-apoptóticos de esta familia, que incluyen Bcl-2, Bcl-xL, Bcl-w, A1/Bfl-1 y Mcl-1 comparten 4 dominios de homología Bcl-2. El rasgo peculiar que se muestra en este grupo es la presencia del dominio BH4 en el N-terminal, a través del cual los distintos miembros de la familia anti-apoptótica de Bcl-2 se unen a las proteínas pro-apoptóticas de la familia Bcl-2, evitando así la activación. Al hacerlo, Bcl-xL respalda la supervivencia celular inhibiendo la vía de muerte celular intrínseca. Como el dominio BH4 es capaz de unirse a otras proteínas que no pertenecen a la familia Bcl-2, algunos miembros anti-apoptóticos pueden contribuir específicamente a otras funciones celulares importantes, como la proliferación, la autofagia, la diferenciación, la reparación del ADN, la progresión tumoral y la angiogénesis.

Entre los distintos miembros de la familia destacamos Bcl-xL, cuya expresión se ha comprobado estar relacionada con varias formas de melanoma metastático. Además de su papel bien conocido en las vías canónicas como la apoptosis y la supervivencia en el cáncer, Bcl-xL también afecta a otras vías, como la invasión/migración, la transición mesenquimatosa

epitelial, y la metástasis. Aunque no hay explicaciones claras de como Bcl-xL podría conferir quimio-resistencia, la agresividad inducida por una sobreexpresión de Bcl-xL se ha asociado a menudo con su capacidad para inducir resistencia a fármacos en cáncer de diferentes orígenes. En el melanoma la sobreexpresión de Bcl-xL está relacionada también con el grosor del tumor y la tasa mitótica. En los últimos años se han desarrollado distintas dianas terapéuticas contra Bcl-xL. Entre ellos, i) oligonucleótidos anti-sentidos, ii) miméticos de las proteínas BH3 capaces de anular la expresión de miembros antiapoptóticos e iii) inhibidores de primera clase.

En los últimos años diferentes estudios han reforzado la idea de que diferentes componentes que rodean al tumor son responsables de su comportamiento, generando el denominado microambiente tumoral (TME). En este entorno las células tumorales pueden crecer y proliferar y al mismo tiempo esconderse del sistema inmunitario. Los componentes de TME son células residentes en tejidos y una gran población de células inmunitarias que se reclutan, entre las cuales se destacan los macrófagos el cuyo papel se ha demostrado ser importante en la promoción de la progresión tumoral hacia una agresividad creciente.

La interacción entre las células cancerosas y los componentes de TME ocurre a través de dos vías principales: ya sea a través de un mecanismo de contacto directo entre las células cancerosas y otra célula o con la matriz extracelular, o a través de la liberación de diferentes moléculas solubles como citoquinas, lípidos mediadores y factores de crecimiento. A parte de los macrófagos otros componentes que contribuyen al mantenimiento y proliferación son: la vasculatura, los fibroblastos asociados al cáncer y la matriz extracelular.

Como ya fue mencionado, los macrófagos juegan un papel fundamental en el microambiente tumoral constituyendo hasta el 50% de una masa tumoral. Los macrófagos con

un papel promotor de tumores normalmente se clasifican como macrófagos asociados a tumores (TAM), cuya presencia en el TME siempre se asocia con un mal pronóstico en la mayoría de los cánceres humanos. El reclutamiento de macrófagos en el sitio del tumor promueve el crecimiento tumoral, estimula la angiogénesis tumoral y facilita la metástasis, lo que permite que la célula tumoral migre y cree un entorno favorable. Una de las principales características de los macrófagos es su capacidad de cambiar su fenotipo según los estímulos que reciben, mostrando una gran plasticidad y heterogeneidad. De acuerdo con la clasificación común, los macrófagos pueden clasificarse como macrófagos M1 que desempeñan un papel antitumoral con propiedades fagocíticas y citotóxicas, y M2 que facilitan el crecimiento tumoral.

Los macrófagos asociados a tumores muestran un fenotipo M2 que deriva de una serie de estímulos provenientes del TME, y de las propias células tumorales capaces de liberar citoquinas, garantizando a los macrófagos mantener un estado continuo de inflamación responsable del fenotipo pro-tumoral.

En los últimos años el pez cebra se ha caracterizado como modelo útil para estudiar el cáncer y la patobiología del melanoma. Al igual que otros vertebrados, el pez cebra desarrolla cánceres benignos y malignos con características histológicas, moleculares y patológicas como los cánceres humanos.

Las ventajas que hacen del pez cebra un modelo complementario atractivo son el rápido desarrollo fuera de la madre, la transparencia en las etapas embrionaria y larvaria, el alto número de progenie, la posibilidad de obtener imágenes en vivo, y el cribado de compuestos. Además de compartir más del 80% de todos los genes relacionados con enfermedades humanas, lo que sugiere la posibilidad de modelar diferentes enfermedades humanas. La

ventaja del uso del pez cebra en melanoma se refleja sobre todo en la simplicidad de visualizar el desarrollo del melanoma. El uso de promotores específicos de tejido para el linaje de melanocitos puede ayudar a visualizar los progenitores de melanocitos y la diferenciación en el embrión vivo y se han utilizado para expresar oncogenes y genes supresores de tumores en estas células. Además, se demostró que las células de melanoma humano inyectadas en embriones de pez cebra de dos días sobreviven, proliferan, migran y forman masas tumorales. Además, debido a la transparencia de los embriones de pez cebra y su desarrollo externo, se pueden realizar estudios para evaluar la influencia del medio ambiente en las células de melanoma y viceversa.

Por otro lado, el modelo de ratón representa el modelo preclínico más utilizado en investigación, debido a algunas de sus características como su fácil manipulación y disponibilidad y la base de conocimiento existente sobre su genética. En los últimos años se han desarrollado diferentes tipos de modelos de ratón, incluidos modelos de xenoinjerto, de ingeniería genética y singénicos. Entre los varios mecanismos utilizados, el xenotrasplante de células de melanoma humano en ratones inmunodeficientes representa una estrategia útil para investigar vías claves del desarrollo y la progresión del tumor. El uso de células derivadas de pacientes (PDTX) representa una ventaja a la hora de mantener más similitudes con los antecedentes de los pacientes. Otro modelo que se ha desarrollado es el trasplante de xenoinjerto singénico que implica la inducción y el trasplante de células de melanoma en la misma especie y antecedentes genéticos. La elección de este tipo de trasplante está asociada a la posibilidad de evaluar la respuesta inmunitaria una vez trasplantadas las células de melanoma y su interacción con el sistema inmunitario. Finalmente, los modelos modificados genéticamente (GEM) llevan una expresión génica modificada útil para la determinación de

los mecanismos de tumorigénesis. La importancia de este modelo se correlaciona con la posibilidad de dilucidar la función de un gen específico e identificar dianas terapéuticas.

El objetivo general de este trabajo es estudiar el papel no canónico que juega Bcl-xL en la interacción entre células de melanoma y los TAMs.

Resultados

A partir de hallazgos previos que demuestran que Bcl-2 específico de melanoma induce el reclutamiento y la polarización de macrófagos a un fenotipo M2, investigamos el posible papel ejercido por la sobreexpresión de Bcl-xL en la regulación del estado de macrófagos mediante el uso de dos diferentes modelos animales, es decir, ratón y pez cebra. En el ratón, tras la inyección subcutánea de células de melanoma en ratones inmunodeficiente, se analizó la infiltración de macrófagos mediante análisis inmunohistoquímico (IHC). Después de treinta días se observó un número significativamente mayor de macrófagos M2 en xenoinjertos que sobreexpresan Bcl-xL en comparación con los de control.

Para confirmar estos datos en un modelo animal diferente, aprovechamos el uso del ensayo de xenotrasplante en embriones de pez cebra. Específicamente, se implantaron células de melanoma parentales y que sobreexpresan Bcl-xL en el saco vitelino de larvas de pez cebra de 2dpf de la línea transgénica *Tg(mpeg1:mCherry; tnfa:eGFP)*, luego se analizó la polarización de las células de los macrófagos en la cola 4 días después de la inyección. Al igual que en el ratón, se evidenció un aumento significativo de la polarización de los macrófagos a un fenotipo M2 en las larvas.

Para comprobar que Bcl-xL era capaz de inducir la polarización de los macrófagos, macrófagos humanos derivado de monocitos, obtenido de capas leucocitarias de donantes sanos, se estimuló con el medio de cultivo (CM) del control o de dos líneas celulares de melanoma que sobreexpresan Bcl-xL. Observamos una expresión significativamente mayor de marcadores M2 (CD163, CD206, CCL1, CCL22 y ARG-1), paralela a una expresión significativamente menor de marcadores M1 (IL-12, CD86, iNOS y HLA) en macrófagos expuesto a CM de células que sobreexpresan Bcl-xL, en comparación con CM de células control. Se obtuvieron resultados similares cuando los monocitos THP-1 se expusieron a diferentes CM. En consecuencia, M-DM estimulada con CM de transfectantes Bcl-xL mostró un aumento de la secreción de TGF-β. También evaluamos la capacidad de polarización M2 de CM de A375 (línea celular de melanoma con altos niveles de Bcl-xL basal/endógeno) silenciada para Bcl-xL (A375 siBcl-xL). El siBcl-xL bloqueó parcialmente la polarización de M2 inducida por A375, provocando una reducción de los marcadores M2, tales como CD163, CD206, CCL1 y CCL22.

También se utilizaron CM de células de melanoma de control y que sobreexpresan Bcl-xL para evaluar su efecto sobre la migración de macrófagos. Se detectó un número significativamente mayor de células migratorias cuando los macrófagos se expusieron a CM derivadas de células que sobrexpresaban Bcl-xL y se compararon con CM de las de control. Se obtuvieron resultados similares cuando los monocitos THP-1 se expusieron a diferentes CM.

Para investigar el posible mecanismo que subyace al proceso de polarización y migración de macrófagos inducido por Bcl-xL, realizamos una matriz de proteínas que compara el CM derivado del control y las células que sobreexpresan Bcl-xL. Se encontró que el nivel de varias

moléculas estaba modulado hacia arriba o hacia abajo en el CM de las células que sobreexpresaban Bcl-xL, en comparación con el CM de las de control. Entre las moléculas reguladas, confirmamos una mayor expresión de ARNm y de proteína de IL-8, IL-1β, IL-1α, TNFα, IL-2, IL-4 e IL-6, y el factor de reclutamiento CCL5/RANTES. Entre los otros factores quimiotácticos de macrófagos más allá de CCL5, el factor estimulante de colonias de macrófagos (MCSF/CSF1), que no está presente en la matriz de proteínas, también se ha encontrado regulado al alza en el nivel de ARNm después de la expresión forzada de Bcl-xL.

Usando anticuerpos de bloqueo específicos, a continuación, centramos nuestra atención en el papel de IL-8 e IL-1β en la polarización de macrófagos y el de CCL5 en la migración de macrófagos. La inhibición de IL-8 provocó una inhibición significativa de los marcadores M2 CD163, CD206 y CCL1, mientras que la inhibición de IL-1β provocó una disminución significativa de los marcadores de macrófagos M2 CD163 y CCL1, y un aumento significativo de los marcadores de macrófagos M1 CD86 y HLA. Es importante destacar que tanto los macrófagos como las células THP-1 mostraron una migración significativamente reducida cuando se expusieron a CM de células de melanoma que sobreexpresan Bcl-xL tratadas con anticuerpos bloqueadores de CCL5 y en comparación con las células expuestas a CM de Bcl-xL células que sobreexpresan tratadas con siRNA de control. El efecto del silenciamiento de CCL5 por siRNA en las células que sobreexpresan Bcl-xL se ha evaluado en la capacidad de las células de melanoma de reclutar macrófagos in vivo. Mientras que las células de melanoma que sobreexpresan Bcl-xL silenciadas para CCL5 no mostraron menos capacidad para invadir la cola de las larvas de pez cebra, la inhibición de CCL5 redujo significativamente la capacidad de los macrófagos para ser reclutados en el sitio del tumor, lo que resultó en interacciones reducidas entre células tumorales invasoras y macrófagos.

Como los genes regulados al alza (IL-8, IL-1β, MCSF y CCL5) en las células de melanoma que sobreexpresan Bcl-xL tienen en común que todos están regulados por NF-kB, evaluamos si este factor de transcripción era el intermediario entre Bcl-xL y la liberación de estas citocinas. Con este propósito, bloqueamos la translocación nuclear de p65 transfectando Bcl-xL que sobreexpresan células de melanoma con IKBSR, la forma mutada de IKB, que actúa como un súper represor de NF-kB, y probamos el efecto sobre la polarización y migración de macrófagos. La inhibición de NF-kB fue capaz de reducir la expresión de IL-8, IL-1β y CCL5 tanto a nivel de ARNm como de proteína. También evidenciamos una capacidad significativamente reducida de los macrófagos y de las células THP-1 para migrar en respuesta a CM de células que sobreexpresan Bcl-xL transfectadas con IKBSR. Se evaluó también el efecto de la inhibición de NF-κB sobre la capacidad de las células de melanoma que sobreexpresan Bcl-xL para invadir la región caudal de las larvas de pez cebra de la línea transgénica con macrófagos marcados en verde *mpeg1: eGFP*. La inhibición de NF-kB pudo reducir la capacidad invasiva de las células de melanoma Mxl90 en comparación con las células Mxl90 control, con una tasa de invasión similar a la de las células de melanoma Mneo originales. Sorprendentemente, la inhibición de NF-kB en células de melanoma que sobreexpresan Bcl-xL también redujo el número de macrófagos reclutados, fenocopiando los efectos de la inhibición de CCL5.

Para explorar el efecto de la ablación de macrófagos en la invasión de melanoma en el modelo de xenoinjerto de pez cebra, aprovechamos la línea *Tg(mpeg1:GAL4FF, UAS:NTR-mCherry)* que expresa la nitrorreductasa bacteriana (NTR) fusionada con mCherry en macrófagos, lo que permite la conversión del profármaco metronidazol (MTZ) en un compuesto citotóxico que provoca la muerte de los macrófagos. La eliminación de los macrófagos redujo la capacidad invasiva de las células de melanoma que sobreexpresan Bcl-

xL a los niveles observados en las células parentales de Mneo. Como era de esperar, MTZ no tuvo efecto alguno sobre la migración de células de melanoma en larvas de tipo salvaje que no expresaron NTR.

Los resultados obtenidos con este trabajo llevan a las siguientes conclusiones:

1) La sobreexpresión de Bcl-xL en células de melanoma promueve el reclutamiento de macrófagos en el tumor y la polarización M2 en modelos de ratón y pez cebra.

2) Los macrófagos derivados de monocitos humanos y las células THP-1 monocíticas se polarizan a un fenotipo M2 cuando se exponen al medio de cultivo de células de melanoma que sobreexpresan Bcl-xL. Además, el medio de cultivo de células de melanoma que sobreexpresan Bcl-xL también promueve la migración de macrófagos derivados de monocitos humanos.

3) El medio de cultivo de células de melanoma que sobreexpresan Bcl-xL induce la polarización M2 in vitro y la migración de macrófagos derivados de monocitos humanos y células THP-1 monocíticas.

4) Las células de melanoma que sobreexpresan Bcl-xL ejercen su efecto sobre el reclutamiento y polarización de macrófagos a través de la liberación de citocinas. Mientras que la IL-8 y la IL-1β están implicadas en la polarización de los macrófagos, la quimiocina CCL5/RANTES media el reclutamiento de macrófagos en el tumor.

5) La regulación de citocinas por Bcl-xL en células de melanoma depende de NF-κB. Por lo tanto, la inactivación genética de NF-κB da como resultado una liberación reducida de citocinas y una migración de macrófagos en modelos de melanoma humano y de pez cebra.

6) La eliminación de los macrófagos en los modelos de xenoinjerto de pez cebra da como resultado una capacidad invasiva reducida de las células de melanoma que sobreexpresan Bcl-xL.

ANNEXE I: PUBLICATION CONTRIBUTION DURING THE PhD

Publications derived from the thesis

- Lucianò, Anna Maria et al. “Exploring association of melanoma-specific Bcl-xL with tumor immune microenvironment.” Journal of experimental & clinical cancer research : CR vol. 42,1 178. 24 Jul. 2023, doi:10.1186/s13046-023-02735-9
- Lucianò, Anna Maria et al. “Bcl-xL: A Focus on Melanoma Pathobiology.” International journal of molecular sciences vol. 22,5 2777. 9 Mar. 2021, doi:10.3390/ijms22052777

Other publications non-related to the thesis

- Lucianò, Anna Maria et al. “The Combination of the M2 Muscarinic Receptor Agonist and Chemotherapy Affects Drug Resistance in Neuroblastoma Cells.” International journal of molecular sciences vol. 21,22 8433. 10 Nov. 2020, doi:10.3390/ijms21228433
- Lucianò, Anna Maria, and Ada Maria Tata. “Functional Characterization of Cholinergic Receptors in Melanoma Cells.” Cancers vol. 12,11 3141. 27 Oct. 2020, doi:10.3390/cancers12113141
- Lucianò, Anna Maria et al. “Effects mediated by M2 muscarinic orthosteric agonist on cell growth in human neuroblastoma cell lines”. Pure and Applied Chemistry Journal https://doi.org/10.1515/pac-2018-1224.

ANNEXE II: CONTRIBUTION TO SCIENTIFIC CONFERENCES DURING THE PhD

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