3-27-2019

The Immunosuppressive Role of Edn3/Ednrb Signaling in the Melanoma Microenvironment

Juliano Tiburcio de Freitas
Florida International University, jfrei022@fiu.edu

Follow this and additional works at: <https://digitalcommons.fiu.edu/etd>

Part of the [Cancer Biology Commons](#), [Immune System Diseases Commons](#), and the [Immunity Commons](#)

Recommended Citation

Tiburcio de Freitas, Juliano, "The Immunosuppressive Role of Edn3/Ednrb Signaling in the Melanoma Microenvironment" (2019). *FIU Electronic Theses and Dissertations*. 4029.
<https://digitalcommons.fiu.edu/etd/4029>

This work is brought to you for free and open access by the University Graduate School at FIU Digital Commons. It has been accepted for inclusion in FIU Electronic Theses and Dissertations by an authorized administrator of FIU Digital Commons. For more information, please contact dcc@fiu.edu.

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

THE IMMUNOSUPPRESSIVE ROLE OF EDN3/EDNRB SIGNALING IN THE
MELANOMA MICROENVIRONMENT

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Juliano Freitas

2019

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Juliano Freitas, and entitled The Immunosuppressive Role of Edn3/Ednrb Signaling in the Melanoma Microenvironment, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

Lou Kim

Mauricio Rodriguez-Lanetty

Anthony McGoron

Marta Torroella

Lidia Kos, Major Professor

Date of Defense: March 27, 2019

The dissertation of Juliano Freitas is approved.

Dean Michael R. Heithaus
College of Arts, Sciences and Education

Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2019

DEDICATION

I dedicate this dissertation to my mother Veronica who fearlessly beat breast cancer in 2015 and my grandmothers Cecilia and Maria who lost their battle against cancer, but their legacy is still alive. Their fights inspired me to pursue a path that seeks to improve cancer patients' lives.

ACKNOWLEDGMENTS

Foremost, I would like to express my gratefulness to the several people who, directly or indirectly, supported me during the six and half years of my Ph.D. I am grateful to God for giving me the knowledge, ability, and strength to persevere and complete this work satisfactorily.

I would like to acknowledge the Department of Biological Sciences for the Teaching Assistantship and the College of Arts, Sciences and Education (CASE) for the Doctoral Evidence Acquisition (DEA) Fellowship that supported my studies. I gratefully acknowledge the Biomedical Research Initiative (BRI) Summer Research Award (NIH/NIGMS R25 GM061347) for the financial support in my work. I would like to recognize the Graduate & Professional Student Committee (GPSC), Department of Biological Sciences and CASE for the funding that made possible my travel to Conferences and Workshops.

I would like to express my deepest appreciation to my advisor Dr. Lidia Kos for guiding me throughout this long journey. I consider myself blessed for having such a clever and inspiring person by my side. Thank you for giving me the opportunity to join your laboratory and always encouraging, especially during the many times my experiments and hypothesis failed. Thank you for showing me that an unexpected result does not mean you are an unskilled investigator, but it is actually a new chance to grow as a scientist. Thank you for making the transition of moving from Brazil so smoothly and providing me with the best possible environment to flourish as the researcher I always dreamed to be. Thank you for always finding at

least a few minutes in your busy schedule to meet with me and provide valuable feedback on my experiments. I would also like to extend my deepest gratitude to the other members of my graduate committee Dr. Lou Kim, Dr. Mauricio Rodriguez-Lanetty, Dr. Anthony McGoron and Dr. Marta Torroella for generously offering their time, support and guidance to improve my work.

I would like to extend my sincere thanks to my lab mates Xiaoshuang Li, Amy Saldana Tavares, Ana Paula Benaduce, Natasha Fernandez, Javier Pino and Nikeisha Chin for sharing the technical assistance that was needed when I first joined in the lab and for all constructive criticism and advice. I am also grateful to the undergraduate students Jessie Sportsman, Joseph Palmer and Martina Cavallini who worked with me during my first project. I am a better mentor thanks to you. I would like to give a special thanks to Jesus Lopez and Claudia Llorian with whom I worked more closely during the last 2-3 years. Your hard work made the completion of this project possible. I would like to recognize the technical support that I received from Dr. Erasmo Pereira as well as his unwavering guidance during this journey. I would like to acknowledge the assistance of Dr. Mariana Boroni on the Bioinformatics analysis.

I am deeply indebted to Dr. Lionel Larue, who opened the doors to his laboratory in France, where I worked as an intern for a month. Although short, this period of time was a game-changer in my career. In your lab I had the chance, for the first time, to use equipment and lab supplies that I only knew from papers. You also highly recommend me to Dr. Kos and convinced me to pursue a Ph.D. in the

United States rather than in Europe. I am also grateful to Dr. Marcelo Vilela (*in memorian*) who gave me the chance to join his lab as an undergraduate student in Brazil. Despite limited funds he inspired me and showed how fascinating science can be.

My success would not have been possible without the support of my lovely wife Ivy. Thank you for staying late on campus for so many times waiting for the final step in the experiment that was supposed to last 5-10 min and ended up taking me an hour or so. Thank you for watching me practicing to my presentations. You are the best support system I could wish for and you have never let me down. You gave up on a promising career in France to follow me in this unbelievable idea to pursue a Ph.D. in the United States. Since we first met in high school you always believed in my potential. You were by side during my happy days but most importantly during my (numerous) bad days. Thank you for believing in me when even myself could not do so. I am also extremely grateful to my beloved parents Cesar and Veronica. Thank you for your prayers, advices, love, and support throughout my life. You always told me education would bring me anywhere. Today I understand you were right. You spared no effort to providing me with the best education possible. I am grateful to my sister Jessica for being a great friend and for filling the emptiness in Dad and Mom's hearts while I was far away. I cannot forget my Brazilian friends Vinicius Paixão, Hudson Balbino, Isabella Carvalho, Faviane Teixeira and Lorena Marçal who, even miles away, cheered me on, and celebrated each accomplishment.

ABSTRACT OF THE DISSERTATION

THE IMMUNOSUPPRESSIVE ROLE OF EDN3/EDNRB SIGNALING IN THE
MELANOMA MICROENVIRONMENT

by

Juliano Freitas

Florida International University, 2019

Miami, Florida

Professor Lidia Kos, Major Professor

Endothelins are cytokines ubiquitously expressed in the microenvironment of several tumors. In melanoma is not clear whether stromal cells respond to the endothelin present in the microenvironment. To address this question, I generated tumors derived from different murine melanoma cell lines (B16F10, YUMM1.7, YUMMER1.7) in a transgenic mouse that overexpresses endothelin 3 (Edn3) by keratinocytes in the skin (*K5-Edn3*). Tumors from all the cell lines grew larger and were more aggressive when Edn3 was overexpressed in the skin. In tumors derived from YUMM1.7-GFP cells, very few tumorigenic cells expressed the Edn3 receptor, Endothelin receptor b (Ednrb) suggesting an environmental role for endothelin signaling in melanoma microenvironment. The present study showed for the first time that Ednrb is expressed in several cell populations inside melanoma microenvironment. The clinical relevance of the finding was validated using publicly available RNA-seq data from melanoma patients. Regulatory T cells

(Tregs) was the only population that was numerically different when *K5-Edn3* tumors were compared to wild-type tumors suggesting that Edn3 promotes Treg enrichment in melanoma microenvironment.

The present study supports the notion that endothelin promotes the formation of an immunosuppressive milieu in melanoma facilitating escape from tumor immunity. Endothelin was required for immune escape of highly immunogenic melanoma cells YUMMER1.7. The immunosuppressive features of Tregs were enhanced in presence of Edn3. The master regulator of Tregs' suppressive functions, FOXP3, was remarkably upregulated in *K5-Edn3* tumors as well as immunosuppressive cytokines TGF- β and IL-10. Overexpression of Edn3 in the tumor microenvironment prevented the expansion of CTLs possibly via GZB-mediated cytotoxicity. Expression of chemokines and inflammatory cytokines were downregulated in *K5-Edn3* tumors. Cytokines that elicit anti-tumor immunity, exhibited reduced levels in *K5-Edn3* tumors. The YUMMER1.7-GFP tumors exposed to high levels of Edn3 were sensitive to immune checkpoint inhibitor (anti-CTLA-4) as well as to Ednrb blockage (BQ-788). The response to BQ-788 was more pronounced suggesting that EDNRB targeting might be an alternative therapeutic strategy for melanoma patients that are under immunotherapy regimen.

In conclusion, the present study indicates that Edn3/Ednrb signaling has an important role in the melanoma microenvironment where it mediates immunosuppression resulting in escape from tumor immunity.

TABLE OF CONTENTS

CHAPTER	PAGE
1.	Introduction..... 1
1.1.	Melanoma..... 2
1.1.1.	Melanoma Biology 2
1.1.2.	Genetic alterations during melanoma development 4
1.1.3.	Melanoma Metastasis 8
1.2.	Tumor Microenvironment..... 10
1.2.1.	Cell types 14
1.2.2.	Cytokines 23
1.2.3.	Melanoma Microenvironment..... 29
1.3.	Endothelin..... 35
1.3.1.	Endothelin Physiology and Pathology 36
1.3.2.	Endothelin signaling in melanocytes and melanoma cells .. 38
1.3.3.	EDNRB and melanoma: clinical opportunities..... 41
1.4.	Research Questions 47
1.5.	Figures..... 49
1.6.	References 57
2.	Expression of Ednrb in Melanoma Microenvironment 78
2.1.	Introduction..... 79
2.2.	Results..... 83
2.2.1.	Endothelin increases tumor aggressiveness..... 84
2.2.2.	Melanoma cells express Ednrb 85
2.2.3.	Ednrb expression in the melanoma microenvironment 86
2.2.4.	Ednrb expression in human melanoma..... 88
2.3.	Discussion 89
2.4.	Methods..... 96
2.4.1.	Mice and genotyping 96
2.4.2.	Cell lines 97
2.4.3.	Tumor cell injection 97
2.4.4.	Flow Cytometry 98
2.4.5.	Histological and Immunofluorescence Analysis 99
2.4.6.	Publicly available RNA-seq data of melanoma samples ... 100
2.5.	Figures..... 101
2.6.	References 111
3.	Immunosuppressive role of Edn3 in the Melanoma Microenvironment 117
3.1.	Introduction..... 118
3.2.	Results..... 125
3.2.1.	Endothelin is required for immune escape of YUMMER1.7 cells..... 125

3.2.2.	Endothelin increases the expression of Tregs-associated markers	126
3.2.3.	Ednrb ⁺ Tregs suppress cytotoxic cells	127
3.2.4.	Immune checkpoint and Ednrb inhibitor decreased melanoma growth.....	127
3.2.5.	Endothelin suppresses expression of inflammatory proteins in melanoma TME	128
3.3.	Discussion	129
3.4.	Methods.....	138
3.4.1.	Tumor cell injection	138
3.4.2.	In vivo experiments	138
3.4.3.	Real-time Quantitative PCR (qRT-PCR)	139
3.4.4.	Western Blotting.....	139
3.4.5.	Immunofluorescence Analysis	140
3.4.6.	IL-10 ELISPOT.....	140
3.4.7.	Cytokine array.....	141
3.5.	Figures.....	142
3.6.	References	152
4.	Conclusions, Implications and Future Directions.....	161
4.1.	Conclusions and Implications	162
4.2.	Future Directions	167
4.3.	Figure	170
4.4.	References	171
VITA.		175

LIST OF FIGURES

FIGURE	PAGE
Figure 1.1 Melanoma progression pathways	49
Figure 1.2 Cells types and signaling molecules in the tumor microenvironment (TME)	50
Figure 1.3 Macrophage polarization	51
Figure 1.4 Naïve CD4+ T cell differentiation	52
Figure 1.5 Tumor inhibitory cytokine network	53
Figure 1.6 Cellular targets of immunosuppressive cytokines	54
Figure 1.7 Endothelin and inflammation	55
Figure 1.8 Endothelin signaling activation in melanoma cells	56
Figure 2.1 Mechanisms of action of endothelin in the TME	101
Figure 2.2 Melanoma cell line and mouse model	102
Figure 2.3 Endothelin overexpression increases aggressiveness of subcutaneous YUMM1.7-GFP melanoma tumors	103
Figure 2.4 Endothelin overexpression increases aggressiveness of intradermal YUMM1.7-GFP melanoma tumors	104
Figure 2.5 Endothelin overexpression increases aggressiveness of intradermal B16F10-GFP melanoma tumors	105
Figure 2.6 Ednrb is expressed in melanoma cells	106
Figure 2.7 Populations of stromal cells present in the melanoma TME	107
Figure 2.8 Ednrb is expressed by stromal cells in the melanoma TME	108
Figure 2.9 Ednrb is expressed by a variety of cell types in the melanoma TME	109
Figure 2.10 EDNRB is expressed by a variety of cell types within the human melanoma TME	110
Figure 3.1 Immunosuppressive mechanisms of Treg in TME	142

Figure 3.2 Endothelin is required for immune escape of YUMMER1.7 melanoma cells.....	143
Figure 3.3 Endothelin increases the expression of Tregs-associated markers in the YUMM1.7-GFP melanoma tumors.....	144
Figure 3.4 Endothelin increases FOXP3 expression in YUMM1.7-GFP melanoma tumors.....	145
Figure 3.5 Endothelin increases TGF- β expression in YUMM1.7-GFP melanoma tumors.....	146
Figure 3.6 Endothelin increases IL-10 expression in YUMM1.7-GFP melanoma tumors.....	147
Figure 3.7 Endothelin increases granzyme B (GZB) expression in YUMM1.7-GFP melanoma tumors	148
Figure 3.8 Endothelin increases the number of Ednrb+ Tregs relative to cytotoxic cells in YUMM1.7-GFP melanoma tumors.....	149
Figure 3.9 Immune checkpoint and Ednrb inhibitor decreased melanoma growth.....	150
Figure 3.10 Endothelin suppress the expression of inflammatory proteins in YUMM1.7-GFP melanoma tumors	151
Figure 4.1 Microenvironmental role of Edn3/Ednrb signaling in melanoma	170

ABBREVIATIONS AND ACCRONYMS

ADC	Antibody-drug conjugated
APC	Antigen presenting cell
ARG-1	Arginase 1
BBB	Blood-brain barrier
BRAF	B-raf proto-oncogene serine/threonine protein kinase
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
CCL2	C-C Motif Chemokine Ligand 2
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD11b	Cluster of differentiation 11b
CD11c	Cluster of differentiation 11c
CD25	Cluster of differentiation 25
CD28	Cluster of differentiation 28
CD45	Cluster of differentiation 45
CD140a or PDGF-R	Platelet-derived growth factor receptor

CD146	Cluster of differentiation 146
CDK2NA.....	Cyclin dependent kinase inhibitor 2A
COX-2	Cyclooxygenase 2
CSD	Chronically sun damaged
CTLA-4	Cytotoxic T lymphocyte associated protein 4
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cell
DMSO.....	Dimethyl sulfoxide
DNA.....	Deoxyribonucleic acid
ECM	Extracellular matrix
Edn1	Endothelin 1
Edn2	Endothelin 2
Edn3	Endothelin 3
EdnrA	Endothelin Receptor A
EdnrB	Endothelin Receptor B
EGF	Epidermal growth factor
ELISPOT	Enzyme-linked Immunospot
F4.80 or Emr1	EGF module-containing mucin-like receptor

FGF	Fibroblast growth factor
FMT	Fecal microbiota transplantation
G.....	Gauge
GFP	Green fluorescent protein
GPCR.....	G-protein coupled receptor
GZB	Granzyme B
H ₂ O ₂	Hydrogen peroxide
HGF	Hepatocyte growth factor
HIF-1 α	Hypoxia-inducible factor-1 α
ICAM-1	Intercellular Adhesion Molecule 1
IFN- γ	Interferon- γ
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10

IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-28	Interleukin 28
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
K5	Keratin 5
Ly6G	Lymphocyte antigen 6 complex locus G
mAB	monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor
MDSC	Myeloid derived suppressor cells
MHC-I	Major histocompatibility complex
mm	Micrometer
mm	Millimeter
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
NF1	Neurofibromatosis type 1
NK	Natural killer

NK1.1	Killer cell lectin-like receptor subfamily B, member 1
NO	Nitric oxide
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
PBS	Phosphate buffer solution
PCR.....	Polymerase chain reaction
PD-1	Programmed cell death-1
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PGE ₂	Prostaglandin E2
PTEN	Phosphatase and tensin homolog
RPM	Revolutions per minute
RT-PCR	Real time-polymerase chain reaction
TAA	Tumor-associated antigen
TAMs	Tumor-associated macrophages
TEC	Tumor endothelial cell
TERT	Telomerase reverse transcriptase
TGF- β	Transforming growth factor β
TME.....	Tumor Microenvironment

TNF- α	Tumour Necrosis Factor α
TP53	Tumor protein 53
Tregs	Regulatory T cells
tTA	Tetracycline-sensitive transcriptional activator
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
YUMM	Yale University Mouse Melanoma
α MSH	α -Melanocyte stimulating hormone
μ l	Microliter

CHAPTER

1. Introduction

1.1. Melanoma

Melanoma is a highly metastatic cancer and the number of new cases of melanoma in the United States has been increasing for the last 30 years. According to the American Cancer Society 96,480 new cases will be diagnosed and about 7,230 people will die from melanoma in 2019. Melanoma has an overall mortality rate of approximately 20% and is responsible for 80% of all deaths related to cutaneous tumors. The high mortality rate associated with melanoma is a result of its remarkable metastatic potential (Paluncic *et al.*, 2016).

1.1.1. Melanoma Biology

Melanoma results from the transformation of pigment cells, namely melanocytes. Melanocytes are neural crest-derived cells that during development migrate to the skin where they reside in the basal layer of the epidermis and hair follicles (Colombo *et al.*, 2011). Melanocytes represent a small population within the skin where there are 10 keratinocytes to each melanocyte (Kanitakis, 2002). Melanocytes are specialized in the production of melanin, the main natural pigment in the skin, which affords a protective effect to keratinocytes against ultraviolet (UV)-induced DNA damage (Costin and Hearing, 2007). Under UV exposure keratinocytes produce α -Melanocyte stimulating hormone (α MSH), which binds to the melanocortin 1 receptor (MC1R) receptor in melanocytes triggering the production of melanin that is eventually delivered to keratinocytes (Schauer *et al.*, 1994; Chakraborty *et al.*, 1996). Melanocytes can produce two different types of melanin: eumelanin, a dark-brown pigment that protects against UV damage and pheomelanin, a yellow-red pigment that is less protective. Individuals with red hair

and fair skin phenotype harbor MC1R variants and therefore are more susceptible to the deleterious effects of UV radiation (Lo and Fisher, 2014).

The major risk factor for melanoma is sun exposure which is represented by the astounding number of UV-signature mutations, such as C>T and G>T, present in melanoma tumors (Lawrence *et al.*, 2013). In fact, melanoma has a median number of >10 mutation/megabase of DNA, which makes it the top tumor type with respect to frequency of somatic mutations (Alexandrov *et al.*, 2013; TCGA, 2015). Very intriguing is the observation that many UV-associated alterations are passenger mutations and have no biological effect as opposed to driver mutations which are oncogenic (Hawryluk and Tsao, 2014). Common mutations found in melanoma, namely *BRAF*^{V600E}, *NRAS*^{Q61L}, *KIT*^{V559A}, and *GNA11*^{Q209L}, lack the typical UV-signatures (Hodis *et al.*, 2012). Nevertheless, the fact that common mutations found in melanoma lack UV-signature does not mean sunlight is not implicated in the mutation load characteristic of melanoma (Schadendorf, Fisher, *et al.*, 2015). Secondary mutations can be generated by the interaction of melanin with UV light and indirectly modify the DNA (Noonan *et al.*, 2012). It should be noted though that genetic alterations in tumor suppressor genes like *tumor protein 53 (TP53)*, *phosphatase and tensin homolog (PTEN)*, and *cyclin dependent kinase inhibitor 2A (CDKN2A)* have a high number of UV-induced mutations (Hodis *et al.*, 2012).

Depending on the amount of cumulative UV-exposure melanomas can be classified as non-chronically sun damaged (non-CSD) or chronically sun damaged

(CSD). Non-CSD melanomas are more prevalent in young patients and arise in regions of the body with intermittent sun exposure. Non-CSD melanomas harbor the *BRAF*^{V600E} mutation and a modest level of mutation burden. Older patients that have been continually exposed to UV radiation tend to develop CSD melanomas, which possess an enormous mutation load and carry different genetic alterations, namely *BRAF*^{nonV600E}, *NRAS* and *neurofibromatosis type 1 (NF1)* mutations (Shain and Bastian, 2016).

1.1.2. Genetic alterations during melanoma development

Melanoma development has long been depicted as a linear progression (Figure 1.1) starting from benign lesions such as melanocytic and dysplastic naevi that transform into melanoma *in situ* which eventually evolves to more malignant lesions like invasive and metastatic melanoma (Shain and Bastian, 2016). A melanocytic naevus is characterized by limited proliferation of melanocytes through the constitutive activation of mitogen-activated protein kinase (MAPK) signaling pathway initiated by *BRAF*^{V600E} mutation (Shain *et al.*, 2015). Neavi formation resulting from Braf activation has been elegantly demonstrated by a mouse model in which only melanocytes harbor the *Braf*^{V600E} mutation. These mutant mice developed lesions very similar to melanocytic naevi found in humans (Dankort *et al.*, 2009). It is widely accepted that melanocytic naevus is the benign precursor of non-CSD melanoma because of the fact that both types of lesions share high amounts of *BRAF*^{V600E} mutations (Pollock *et al.*, 2003). On the other hand, CSD melanomas, which have *BRAF*^{nonV600E} mutations and tend to arise from *de novo* lesions, have no evolutionary connections with melanocytic naevi (Shitara

et al., 2014). In the absence of additional genetic alterations melanocytic naevi are directed to a senescence-like stage defined by cell cycle arrest and abrogation of cell proliferation. The *BRAF*^{V600E} mutation is an important trigger of the senescence observed in melanocytic naevi illustrating the well-known oncogene-induced senescence phenomenon (Damsky and Bosenberg, 2017). The immune system may also enhance senescence as activation of BRAF stimulates the secretion of pro-inflammatory mediators interleukin 6 (IL-6) and interleukin-8 (IL-8) (Kuilman *et al.*, 2008). Interestingly, the expression of these inflammatory mediators are augmented in melanocytic naevi when compared to more aggressive lesions (Ahmed *et al.*, 1995).

The vast majority of melanocytic naevi remain dormant and never give rise to melanoma. It is estimated that during a man's lifetime there is a risk of 1/3,000 that a nevus will progress to melanoma and for women the odds are even lower (1/11,000) (Tsao *et al.*, 2003). Melanocytic naevi rarely acquire mutations in the telomerase reverse transcriptase (TERT) promoter and heterozygous alteration in the *CDNK2A* locus, which activate telomerase and disturb the cell cycle checkpoints, respectively (Shain *et al.*, 2015). These alterations result in the formation of an intermediate lesion between benign and malignant melanoma called dysplastic naevus (Elder *et al.*, 1980). Mutations in the TERT promoter are considered one of the earliest secondary mutations present in the naevus and roughly 80% of dysplastic naevi exhibit these alterations (Shain *et al.*, 2015). Individuals with germline variants of *CDNK2A* tend to develop numerous *de novo* dysplastic naevi leading to dysplastic nevus syndrome (Shain and Bastian, 2016),

and have 70% of chance to develop familial melanomas during their lifetime (De Snoo and Hayward, 2005). Some dysplastic naevi developed sporadically have greater number of *NRAS* mutation compared to the amount of *BRAF*^{V600E} mutation present in the melanocytic naevi suggesting that in some case melanocytic and dysplastic naevi do not follow the same evolutionary pathway (Shain *et al.*, 2015). Immune surveillance seems to be tightly associated with emergence and growth of naevi. In a condition termed eruptive naevi individuals abruptly develop numerous naevi harboring the *BRAF*^{V600E} mutation and the phenomenon is particularly common in immunosuppressed patients (Sekulic *et al.*, 2010).

Transformed melanocytes with excessive proliferation restricted to the epidermis is a characteristic of melanoma *in situ*. In addition to the heterozygous mutations in *CDKN2A* and alterations in the TERT promoter *in situ* melanomas have increased mutations affecting downstream effectors of MAPK signaling pathway (Shain and Bastian, 2016). Approximately 50% of *in situ* melanomas have the *BRAF*^{V600E} mutation, and 30% harbor the *NRAS*^{Q161L} mutation. (Omholt *et al.*, 2002, 2003; TCGA, 2015). Interestingly, those mutations are mutually exclusive implying an absence of selective advantage in harboring many alterations in the MAPK signaling pathway (Damsky and Bosenberg, 2017). Inactivation of tumor suppressor gene *NF1*, a negative regulator of RAS, is the third most common genetic alteration in melanomas and is most prevalent in older patients (Krauthammer *et al.*, 2015; TCGA, 2015). There is a fourth subtype of melanoma that does not harbor either of those three classical mutations (*BRAF*, *NRAS* or *NF1*) in the MAPK signaling pathway and therefore is considered triple wild-type

melanoma. Mutations in the receptor tyrosine kinase KIT and in the α subunits of G-protein GNA11 and GNAQ are more frequent in triple wild-type melanomas (TCGA, 2015). *In situ* melanoma may endure for decades gathering a variety of mutations before acquiring the ability of breaking the epidermis and invading the surrounding tissues (Shain and Bastian, 2016).

Once melanoma is able to occupy the dermis it is considered to be at the invasive stage. The level of invasiveness is associated with reduced survival and used as a prognostic factor (Schadendorf, Fisher, *et al.*, 2015). A crucial genetic alteration found in early invasive melanomas is the inactivation of the second copy of *CDKN2A* (Shain *et al.*, 2015). Alternative splicing of *CDKN2A* locus produces two different proteins, p16^{INK4A}, and p14^{ARF} (or p19^{ARF} in mice), that are considered major tumor suppressors in melanoma (Damsky and Bosenberg, 2017). The protein p16^{INK4A} phosphorylates Rb preventing cell cycle progression in G1/S phase (LaPak and Burd, 2014) while p14^{ARF} inhibits p53 degradation by MDM2 (Maggi *et al.*, 2014). A mouse model with the *Braf*^{V600E} mutation in combination with *Cdkn2a* inactivation develops melanomas with 100% of penetrance as mice age (Damsky *et al.*, 2015) and transgenic mice harboring the *Nras*^{Q61K} mutation and homozygous deletion of *Ink4a* gene develop invasive melanomas in six months (Ackermann *et al.*, 2005).

Later in the progression of the primary tumor the phosphatase PTEN is often lost as metastatic melanomas have high frequency of PTEN inactivation (Reifenberger *et al.*, 2000). Silencing of PTEN associated with the *BRAF*^{V600E}

mutation is observed in 80% of melanoma cell lines (Tsao *et al.*, 2004) and 40% of melanoma patients (Hodis *et al.*, 2012). Transgenic mice have confirmed that *Braf*^{V600E} mutation alone is not sufficient to produce invasive melanoma, but when it is associated with the *Pten* deletion, mice develop melanomas with high penetrance that metastasize to lymph nodes and lungs (Dankort *et al.*, 2009). The protein p53 is another tumor suppressor altered during late stages of melanoma (Shain and Bastian, 2016). Disruption of p53 in melanoma is lower compared to other types of solid tumors but mutations in the *TP53* gene are more frequent in metastatic lesions when compared to primary melanomas (Stretch *et al.*, 1991).

1.1.3. Melanoma Metastasis

Melanoma is referred as metastatic after tumorigenic cells leave the primary tumors and disseminate to distant sites in the body. Once melanoma becomes metastatic, the patients has a very poor prognosis with the 1-year overall survival rate around 25% and the median survival of 6.2 months (Korn *et al.*, 2008). Melanoma generally establishes secondary lesions in brain, lymph nodes, skin and lung (Murakami, Cardones and Hwang, 2004), the latter being the most common tissue affected in approximately 40% patients with metastatic disease (Balch *et al.*, 1983). The nearest draining lymph to primary tumors is generally thought to be the first site of dissemination of metastatic cells and the colonization of visceral organs happens afterwards (Shain and Bastian, 2016). The presence of melanoma cells in the sentinel lymph node has long been accepted as prognostic factor for patients (Balch *et al.*, 2009). Yet, the complete resection of the sentinel lymph node does not seem to improve patient survival (Kingham *et al.*, 2010).

Recently it has been suggested that metastasis might be formed earlier in the history of the primary tumor and the idea challenges the classical paradigm of linear progression of melanoma metastasis formation (Gassenmaier *et al.*, 2017). Currently, there are some indications that metastasis formation takes place in parallel to the development of the primary tumor (Damsky, Theodosakis and Bosenberg, 2014; Sanborn *et al.*, 2015), although the molecular mechanisms underlying the parallel progression model are not established yet (Figure 1.1). Patients with no signs of metastatic disease have melanoma cells (Reid *et al.*, 2013) and even benign melanocytes detected in their bloodstream (Hall *et al.*, 2012). In the condition termed nodal naevi, melanocytes carrying *BRAF*^{V600E} mutation are found in lymph nodes (Taube *et al.*, 2009) and nodal naevi have been detected in patients with no melanoma disease (Bautista, Cohen and Anders, 1994). Further observation supporting the parallel progression model of melanoma metastasis includes the fact that 5% of patients with metastatic disease have no detectable primary tumor (Giuliano, Moseley and Morton, 1980). It is reasonable to speculate that metastatic features are established during benign stages of melanoma development rather than acquired during progression, but the development of fully metastatic phenotype would require further mutations gained later during tumorigenesis (Shain and Bastian, 2016). The linear and parallel models of melanoma dissemination are not necessarily mutually exclusive since both pathways are likely to generate relevant metastatic disease (Damsky, Theodosakis and Bosenberg, 2014).

1.2. Tumor Microenvironment

Cancers had long been viewed as an assembly of heterogeneous transformed cells. The reductionist view of tumors has changed over the past decades to a more complex landscape. Presently, a tumor is seen as an “organ” where an elaborate and bidirectional interaction between cancer cells and non-malignant cells from the surrounding stromal tissue takes place (Hanahan and Weinberg, 2011). Inside these “organs” there is a complex network called the **tumor microenvironment (TME)** that includes not only the cancer cells, but also the tumor stroma which is a milieu that comprises non-neoplastic cells, extracellular matrix (ECM), and the signaling molecules they are embedded in (Spano and Zollo, 2012). The cellular component of the TME is diverse and include fibroblasts, endothelial cells, and a variety of immune cell populations namely, macrophages, neutrophils, myeloid derived suppressor cells (MDSC), dendritic cells (DC), natural killer (NK) cells, and T and B lymphocytes subtypes (Quail and Joyce, 2013).

The TME is a not an inert component but rather, a continuously evolving system modulated by oncogenic and environmental signals and, in response to the metamorphic TME, tumor cells are also modified and become more aggressive (Binnewies *et al.*, 2018). Thus, it is now well established that the TME plays a crucial role during tumor development, supporting tumor growth, invasion and metastasis (Spano and Zollo, 2012). In some cancers the malignant cells represent only 30% of all cell types existing in the TME (Becker *et al.*, 2013). Furthermore, the amount of different cell populations in the tumor stroma varies drastically when different tumors are compared or even among patients harboring the same type of

cancer. Indeed, each tumor has its own “TME fingerprint” that can be addressed for prognostic and therapeutic purposes (Liu, Workman and Vignali, 2016).

Tumor formation and development is associated with a deregulated microenvironment as observed by the increased incidence of cancers in individuals that experience chronic inflammation or immunosuppression (Palucka and Coussens, 2016). Patients with long-term colitis have seven times more chance to develop colon cancer than those with no inflammation in their colon (Beaugerie *et al.*, 2013). Lung and skin cancer arise more frequently in organ transplant recipients that are under immunosuppressive regimen (Stewart *et al.*, 1995). Upon the initial transformation of cells in a tissue, the tumorigenic cells recruit cancer-associated fibroblasts (CAFs). Cancer-associated fibroblasts establish a crosstalk with the tumorigenic cells based on chemokines and growth factors, which in turn attract immune cells to participate in the network. Immune cells play ambiguous roles during tumorigenesis, either antagonizing or supporting tumors and the balance between tumor suppressing or promoting immune cells will determine the fate of tumor progression (Spano and Zollo, 2012).

Emerging tumor cells overexpress mutated or new antigens that are collectively called tumor-associated antigens (TAA). Tumor-associated antigens can be recognized by DCs and presented to CD4⁺ Helper T cells, which activate CD8⁺ cytotoxic T cells (CTLs) that will eventually eliminate the cancer cells (Lee, 2017). Cancer cells can also reduce their own immune recognition deregulating MHC-I expression. However, NK cells can detect the missing self in the tumors and

destroy them (Morvan and Lanier, 2016). The elaborate network of immune cells devoted to finding and removing cancer cells is termed tumor immunosurveillance and is constantly monitoring every host tissue and keeping the body clear of cancer cells (Grivennikov, Greten and Karin, 2010; Hanahan and Weinberg, 2011). The tumor immunosurveillance hypothesis is supported by the observation that immunocompromised mice lacking different populations of lymphocytes are more susceptible to induced and spontaneous tumors than their immunocompetent counterparts (Shankaran *et al.*, 2001).

The relationship between cancer and inflammation has long been acknowledged. In the mid nineteenth century, it was already demonstrated that tumors have a high incidence of leukocyte infiltrates (Balkwill and Mantovani, 2001). Since immune cells can detect and destroy cancer cells, the presence of inflammation in neoplastic contexts had been interpreted as a frustrated attempt by the immune system to eliminate the tumor. Some authors described tumors as wounds that never heal (Flier, Underhill and Dvorak, 1986). Counterintuitively, over the past decades it has become clear that inflammation may endow tumorigenesis. The inflammatory milieu is characterized by large amounts of growth, survival and angiogenesis factors, which promote tumor growth, evasion of growth suppression and angiogenesis, respectively. The large number of reactive oxygen species generated during inflammation may also increase the genetic instability through mutagenic events in the tumor cells (Condeelis and Pollard, 2006; Hanahan and Weinberg, 2011).

Inflammation alone is not sufficient for tumor progression because of the intense cell recruitment of immune cells to the TME potentially exposes tumor cells to immune detection and elimination. Many of the inflammatory mediators are also related to the recruitment and activation of immunosuppressive cells such as MSDC and regulatory T cells (Tregs) (Umansky and Sevko, 2012). These cells show remarkable immunosuppressive and anti-inflammatory activities and are able to disturb many mechanisms of immunosurveillance, including but not restricted to the impairment of T cell activation, antigen presentation by DC and interruption of NK cells cytotoxicity (Quail and Joyce, 2013). Therefore, the recruitment of immune cells to the TME is highly advantageous to tumor cells given that inflammatory cells foster tumor progression while immunosuppressive cells make those anti-tumor recruited cells unable to recognize and kill cancer cells (Hanahan and Weinberg, 2011).

Immunoediting explains the contradictory interaction of immune cells in the TME throughout tumor development and progression. The immunoediting concept was derived from the findings that immunocompromised mice develop tumors that are more immunogenic (more easily detected by the immune cells) than tumors arising in immunocompetent mice (Shankaran *et al.*, 2001). According to the immunoediting concept, the host immune system prevents tumor formation while modulating tumor immunogenicity. Immunoediting happens through a sequence of stages starting from elimination, evolving to equilibrium and culminating in the escape phase. The elimination phase is when the immunosurveillance system detects and eradicates emerging tumors. Rarely, tumors cells that subsist the

forces of immunosurveillance are kept dormant during the equilibrium phase. The equilibrium phase is probably the longest phase of the immunoediting process. Throughout equilibrium phase the immune system selects and edits the immunogenicity of tumor cells. During the escape phase tumor cells that became less immunogenic form visible tumors, further enhanced by the immunosuppressive setting installed in TME (Schreiber, Old and Smyth, 2011).

The TME has recently attracted much attention for its therapeutic opportunities. For many years the approach to treat patients with malignant tumors depended solely on targeting tumor cells. Tumors initially respond to targeted therapies but as a consequence of the genetic instability of malignant cells they become resistant to treatments. Presumably, a more effective alternative is to target the stromal cells in the TME. Non-transformed cells are genetically stable and offer reduced opportunity to circumvent targeted treatments (Quail and Joyce, 2013). During the last few years approaches aiming to reprogram the TME and shift the balance toward a more anti-tumorigenic phenotype have considerably improved patients' outcome. Many of these strategies, called immunotherapy, are meant to boost the host's immune system to combat cancer. Immunotherapy has produced exciting results and in the next decade it is likely that 60% of patients with advanced tumors will be treated with immunotherapy (Ledford, 2014).

1.2.1. Cell types

Cells in the TME can be classified as either pro-tumorigenic or anti-tumorigenic. Among pro-tumorigenic cells there are macrophages, neutrophils, T and B cells,

and MDSCs. The anti-tumorigenic cells are CTLs and NK cells (Hanahan and Weinberg, 2011). A more complex classification is defined by the dual role of immune cells in the TME: immune cells with an anti-tumorigenic role are “type 1 cells” whereas those with a pro-tumorigenic role are “type 2 cells”. The CD4⁺ T helper cells T_H1, neutrophils N1, and macrophages M1 are all tumor-inhibitory cells. In contrast, CD4⁺ helper cells T_H2, neutrophils N2 and macrophages M2 are all tumor-promoting cells (Becker *et al.*, 2013). So far, NK cells have not been described as playing pro-tumorigenic roles in the TME (Smith and Kang, 2013). The “type 1” and “type 2” classification of immune cells in the TME is very useful for describing the basic concepts underlying the TME. However, the classification should be viewed with caution because the complexity of TME cannot be reduced to an all or none framework (Quail and Joyce, 2013). Another dichotomous classification of the TME is focused on its global immunological phenotype. Tumors with low number of anti-tumorigenic cells such as CTL, NK and T_H1 lymphocytes, few DCs and high number of immunosuppressive cells (i.e., MSDC and Tregs) are considered “cold” tumors. Conversely, “hot” tumors are highly inflamed and enriched with effector anti-tumor cells and antigen presenting cells (APCs) and therefore are likely to have a better response to immunotherapies (Nagarsheth, Wicha and Zou, 2017; Binnewies *et al.*, 2018). A diagrammatic representation of the many cell types and their interactions in the TME is shown in Figure 1.2.

1.2.1.1. Anti-tumorigenic immune cells

Mature DCs are very efficient APCs that initiate strong anti-tumor response upon interaction with lymphocytes (Nagarsheth, Wicha and Zou, 2017). Dendritic cells activate lymphocytes when TAAs are presented in presence of co-stimulatory signals. The best characterized co-stimulatory signal system is CD28-B7; CD28 is expressed by T cells while B7 is expressed in the DC surface. The protein B7 can also bind to the inhibitory receptor cytotoxic T lymphocyte associated protein 4 (CTLA-4), but in binding to CTLA-4 prevents T cell activation (Andersen *et al.*, 2006). When DCs loaded with TAA encounter naïve CD8⁺ T cells they promote T cell differentiation into TAA-specific CTLs. A similar differentiation process happens when DCs find naïve CD4⁺ T helper cells but in this case T helper cells can differentiate into subpopulations with different phenotypes including T_H1 and T_H2 (Palucka and Coussens, 2016). Dendritic cells induce the polarization of CD4⁺ T helper cells towards T_H1 or T_H2, a process that relies on (interleukin 12) IL-12 availability. Antigen presentation in presence of IL-12 generates T_H1 cells. In contrast, when antigens are presented in absence of IL-12, CD4⁺ T helper cells differentiate into T_H2 cells (Banchereau and Palucka, 2005).

The T_H1 polarized CD4⁺ T helper cells are associated with destruction of malignant cells. A large number of T_H1 cells is correlated with good outcome for patients (Galon *et al.*, 2006). The T_H1 cells work in coordination with DCs to promote the activation of tumor-specific CTLs through cross-presentation of TAAs (Dunn, Old and Schreiber, 2004). Another anti-tumor mechanism of T_H1 cells is via the production of interferon- γ (IFN- γ), which is one of the main modulators of

the host response against tumors (Ikeda, Old and Schreiber, 2002). Interferon- γ derived from T_H1 cells polarizes monocytes towards M1 phenotype (Mantovani *et al.*, 2008) and activates the cytotoxic activity of macrophages as well as CTLs facing tumors cells (Ikeda, Old and Schreiber, 2002; Becker *et al.*, 2013).

Cytotoxic T cells (CTLs) is considered the principal cell type of anti-tumor immunity (Appay, Douek and Price, 2008) and, not surprisingly, tumors with high infiltrates of CTLs are associated with improved prognosis (Clemente *et al.*, 1996; Prall *et al.*, 2004; Bachmayr-Heyda *et al.*, 2013). Cytotoxic T cells have two strategies to kill cancer cells that require direct cell-cell contact. The first mechanism is through the secretion of granules of perforin and granzyme B (GZB) in the extracellular space, the other apparatus is via Fas ligand/Fas receptor. Both approaches trigger apoptosis in the target cancer cell and must be tightly regulated to prevent accidental killing of the CTLs themselves or other neighboring cells. Cytotoxic T cells also secrete cytokines like IFN- γ and TNF- α that similarly activate apoptosis in the target cells (Andersen *et al.*, 2006). Upon long-lasting exposure to TAAs, CTLs might enter in a stage called exhaustion, characterized by reduced proliferation and cytotoxic activity as well as augmented expression of inhibitory receptors such as CTLA-4 and programmed cell death-1 (PD-1). The expression of inhibitory receptor ligands by tumor and stromal cells in the TME prevents CTL activity. Recently, immunotherapy treatments aiming to block the expression of immune checkpoint inhibitors have successfully strengthened tumor infiltrating CLTs and provided durable anti-tumor response in patients with advanced tumors (Zarour, 2016).

Natural killer (NK) cells are cells from the innate immunity system that also have strong cytotoxic activity against tumor cells (Corthay, 2014). High tumor incidence in animal models (Talmadge *et al.*, 1980) and patients with NK cell deficiencies, supports the pivotal role of NK cells in immunosurveillance (Roder *et al.*, 1980). Likewise CTLs, NK cells kill tumors cells using granzymes and perforin granules as well as death receptor/ligand pathways. The NK cells can discriminate between healthy and cancer cells on the basis of the balance between inhibitory and activating receptors. Inhibitory receptors detect self-major histocompatibility complex-I (MHC-I) in normal cells and prevent the activation of NK cells (Morvan and Lanier, 2016). Cancer cells recurrently downregulate MHC-I expression so that they can escape CTL detection. The NK cells detect the missing self on tumors cells that escaped CTLs control and this process triggers NK activity. Some cancer cells maintain MHC-I expression and express ligands to NK's activating receptors (i.e. NKG2D) and the binding of these ligands also stimulate NK cytotoxic activity (Muenst *et al.*, 2016).

1.2.1.2. Pro-tumorigenic immune cells

Tumor-associated macrophages (TAMs) are major contributors to tumor cell migration, invasion and metastasis. They are mainly localized in the tumor edge where they facilitate tumor invasion (Condeelis and Pollard, 2006). Indeed, there is a positive correlation between augmented metastatic incidence and high TAM infiltration (Hanada *et al.*, 2000). Tumor-associated macrophages mainly show M2 phenotype in the TME (Rhee, 2016) and are recognized as the main source of matrix-degrading proteases which promote tumor cell invasion (Quail and Joyce,

2013). In breast and pancreatic cancer, TAMs are associated with production of metalloproteinases (MMP) MMP-2 and MMP-9 (Hagemann *et al.*, 2004) as well as cathepsin B and S secretion (Gocheva *et al.*, 2010). Tumor-associated macrophages also influence tumor invasion through a signaling loop involving EGF and CSF-1. Epidermal growth factor (EGF) secreted by TAMs interacts with its receptor on tumor cells and in addition to cell motility, promotes the tumor secretion of CSF-1, which in turn stimulates TAMs to produce more EGF (Goswami *et al.*, 2005). Additionally, TAMs have reduced antigen presentation capacity and produce immunosuppressive cytokines IL-10 and TGF- β (Muenst *et al.*, 2016).

Myeloid derived suppressor cells (MDSC) are immature cells with potent immunosuppressive features detected in tumors of murine models and patients with different types of tumors. In mice, MDSCs are defined by the co-expression of macrophage (CD11b⁺) and neutrophil (Gr1⁺) markers, but in humans, their characterization is more complex since human MDSCs are comprised of populations with diverse phenotypes (Spano and Zollo, 2012). The MDSCs can be distinguished as either monocytic (M-MDSC), that are more like monocytes, or polymorphonuclear (PMN-MDSC), resembling neutrophils. M-MDSCs have stronger immunosuppressive capabilities than PMN-MDSCs which explains why M-MDSCs are abundant in tumors (Kumar *et al.*, 2016). The MDSCs have different mechanisms to disturb immunosurveillance and orchestrate immunosuppression in the TME. The MDSCs impair T cell activity by the expression of arginase (ARG-1). Arginase consumes L-arginine and the depletion of this essential amino acid from the TME causes downregulation of T cell receptor complex and proliferative

arrest. The expression of iNOS enzyme by MDSCs creates oxidative stress which also affects T cell function (Gabrilovich, Ostrand-Rosenberg and Bronte, 2012). Myeloid derived suppressor cells also prevent M1 polarization (Sinha, Clements and Ostrand-Rosenberg, 2005) and disrupt NK cell cytotoxicity (Liu *et al.*, 2007). Moreover, MDSCs promote the conversion of naïve CD4⁺ T cells into Tregs as well as their expansion, and these processes might be dependent on the production of immunosuppressive cytokines interleukin-10 (IL-10) and transforming growth factor β (TGF- β) (Huang *et al.*, 2006).

Regulatory T cells (Tregs) are immunosuppressive cells that during homeostasis prevent excessive immune responses and promote self-tolerance, but in the context of tumorigenesis, Tregs may allow the escape of tumor cells from immunosurveillance (Lee, 2017). The association of Tregs with patient outcome is very controversial. In many cancers, including melanoma, the accumulation of Tregs is correlated with poor prognosis (Knol *et al.*, 2011). Conversely, when tumor initiation is associated with chronic inflammation (i.e., colorectal cancer) Tregs have a protective effect and therefore high numbers of this immunosuppressive cell predicts improved survival (Frey *et al.*, 2010). Treg cells exert their repressive role onto tumor-specific lymphocytes via a variety of mechanisms including production of immunosuppressive cytokines IL-10 and TGF- β , eliminating IL-2 which is an essential cytokine for CTLs expansion and expressing the immune checkpoint inhibitor CTLA-4, a common target in immunotherapy (Schreiber, Old and Smyth, 2011).

1.2.1.3. Non-immune stromal cells

Cancer-associated fibroblasts (CAFs) are the pioneer cells recruited to the TME and the most numerous cell population in the tumor stroma (Spano and Zollo, 2012). CAFs, sometimes referred as myofibroblasts, are different from normal fibroblasts and detectable by the expression of α -smooth muscle actin (α -SMA) (Egeblad, Nakasone and Werb, 2010). The origin of CAFs present in the TME is still unclear. Some findings have suggested that fibroblasts may arise after epithelial cells experience epithelial-to-mesenchymal transition (EMT) (Petersen *et al.*, 2003), whereas another study presented the possibility of endothelial cells generating fibroblasts after undergoing endothelial-to-mesenchymal transition (EndMT) (Zeisberg *et al.*, 2007). Co-culture of cancer cells with normal fibroblasts or CAFs has demonstrated that CAFs are important for tumor initiation (Olumi *et al.*, 1999) and invasive phenotype (Dimanche-Boitrel *et al.*, 1994). Cancer-associated fibroblasts have many secretory functions that promote tumor growth and are considered the main source of growth factors in the TME (Quail and Joyce, 2013). For example, hepatocyte growth factor (HGF) derived from CAFs provides the oncogenic signal for tumor cell proliferation. In addition, CAFs modify the ECM through the secretion of many ECM components (tenascin C, collagen I and II) as well as matrix metalloproteinases (MMPs) accelerating tumor progression (Kalluri and Zeisberg, 2006). Furthermore, CAFs modulate the recruitment of different populations of immune cells to the TME and ablation of CAFs from the TME reduces the amounts of TAMs, MDSCs and Tregs (Liao *et al.*, 2009). Cancer-associated fibroblasts are also an important supplier of vascular endothelial growth

factor (VEGF) which underscores the pivotal function of fibroblasts during tumor angiogenesis (Fukumura *et al.*, 1998).

Endothelial cells are also a critical cellular component of the TME. Besides their vascular function in supplying nutrients to the TME, they have the capacity to create new routes for recruitment of immune cells and metastatic cells dispersion (Smith and Kang, 2013). Pro-angiogenic factors induce the sprouting of new vessels from existing ones in a process known as angiogenesis (Fang and DeClerck, 2013). In the earlier 1970s it was proposed that tumor development relies on angiogenesis (Sherwood, Parris and Folkman, 1971) and since then angiogenesis has been considered a hallmark of cancer (Hanahan and Weinberg, 2011). Vascular endothelial growth factor is the major regulator of angiogenesis and its production in the TME results in endothelial cell migration and proliferation as well as increased vascular permeability (Hida *et al.*, 2018). Tumor endothelial cells (TECs) are very different from their normal counterparts in many characteristics including gene expression profiles, chromosomal abnormalities and proliferative and migratory features (Maishi and Hida, 2017). Tumor endothelial cells from tumors with different metastatic potential are also distinct suggesting that endothelial cells are involved in metastasis formation (Ohga *et al.*, 2012). When TECs derived from highly metastatic tumors are co-injected with poorly metastatic cancer cells the metastatic potential of these tumor cells is enhanced (Maishi *et al.*, 2016).

1.2.2. Cytokines

The different cell populations present in the TME establish a complex communication network and much of the cross-talk is regulated by cytokines that promote tumor growth and progression (Burkholder *et al.*, 2014). The term cytokine was primarily used to describe the group of secreted molecules derived from hematopoietic cells. A sophisticated concept suggests that cytokines are molecules produced by a variety of cell types and used for cell-to-cell communication. These molecular messengers are major modulators of many physiologic and pathologic events including hematopoiesis, immune response, angiogenesis, wound healing and cancer (Huang *et al.*, 2012). There are two fundamental features of cytokines that influence their microenvironmental action. Cytokines are pleiotropic molecules meaning that the same cytokine can produce diverse effects in different cell types. Cytokines are also redundant molecules, thus the action of different cytokines may have the same outcome in a cell population (Ozaki and Leonard, 2002).

Cytokines play a decisive role during the polarization and biological action of some immune cells present in the TME. An integrated system of cytokines educates neutral and immature immune cells towards tumor-inhibitory or tumor-promoting effector phenotypes. Tumor microenvironment education is a consequence of intrinsic plasticity of immune cells under the influence of stimulatory or suppressive cytokines (Quail and Joyce, 2013). Macrophages and CD4⁺ T cells are particularly susceptible to this molecular communication and in

response to cytokines existing in the TME they differentiate into either anti-tumorigenic or pro-tumorigenic subtypes (Burkholder *et al.*, 2014).

Macrophage polarization has been well-described in many studies. Monocytes, the precursors of macrophages, are very sensitive to the first cytokines they encounter in the TME (Figure 1.3). Upon arrival in the TME, if monocytes are initially exposed to the cytokine IFN- γ they differentiate into M1 macrophages (Grivennikov, Greten and Karin, 2010). Macrophages M1 secrete many pro-inflammatory cytokines, namely IL-1, IL-6, IL-12, and TNF- α (Rhee, 2016). Conversely, if after arriving in the TME monocytes first encounter the cytokines IL-4, IL-10, IL-13 or TGF- β (Allavena *et al.*, 2008) they are polarized towards M2 phenotype and produce anti-inflammatory cytokines TGF- β and IL-10 (Sica, Allavena and Mantovani, 2008). Macrophage phenotype is very plastic therefore it is also possible that, as a result of cytokine re-education, M2 macrophages switch towards M1 phenotype (Quail and Joyce, 2013).

The CD4⁺ helper T cells can be distinguished into many subtypes depending on their cytokine profile (Figure 1.4). The differentiation of naïve CD4⁺ T cell into effector subsets found in the TME is orchestrated by APC-derived cytokines during antigen presentation. Remarkably, the specific cytokines of a subgroup can reinforce its differentiation while prevent the differentiation into a different subtypes (Gutcher and Becher, 2007). Initially, it was believed that naïve CD4⁺ T cells would only be able to polarize towards either T_H1 or T_H2. When APCs present antigen to naïve CD4⁺ T cells in presence of IL-12, T_H1 cells are generated (Seder *et al.*,

1993) and T_H1 cells will prominently secrete IFN- γ in addition to TNF- α , IL-2 and IL-12 cytokines (Burkholder *et al.*, 2014). On the other hand, if there is no IL-12 during antigen presentation, naïve CD4⁺ T cells start to secrete IL-4 and differentiate into T_H2 cells (Swain *et al.*, 1990), which in addition to IL-4 also produce IL-5 and IL-10 cytokines (Moss *et al.*, 2004). Later it was discovered that TGF- β is also an important regulator of naïve CD4⁺ T cell polarization. Secretion of TGF- β alone by APC generates the immunosuppressive subtype Treg that produces IL-10 and TGF- β (Chen *et al.*, 2003). However, when TGF- β is supplemented with IL-6, naïve CD4⁺ T cells polarize towards the pro-inflammatory subtype T_H17 which produces IL-17 (Veldhoen *et al.*, 2006). The T_H17 cells execute their anti-tumor actions recruiting more CTLs, NKs and DCs to the TME (Nagarsheth, Wicha and Zou, 2017).

A cytokine network comprised of the canonical pro-inflammatory cytokines IL-1, IL-6, and TNF- α as well as T_H1-associated cytokines IL-12 and IFN- γ coordinates the effective anti-tumor immune response accountable for tumor immunosurveillance (Figure 1.5). Tumor-specific T_H1 cells produce the proliferative cytokine IL-2 in addition to IFN- γ , a cytokine that polarizes macrophages towards M1 phenotype. Polarized M1 macrophages secrete IL-12, that enhances T_H1 phenotype, and also a series of pro-inflammatory cytokines (i.e., IL-1, IL-6 and TNF- α) that have tumoricidal activity (Haabeth, Bogen and Corthay, 2012). Interleukin-1 cooperates with IL-2 to promote proliferation of CTLs and NK cells (Aribia *et al.*, 1987) and enhances the antigen-presenting functions of DCs (Burkholder *et al.*, 2014). Interleukin-6 increases CD4⁺ and CTL anti-tumor

activity and prevents tumor progression (Mulé *et al.*, 1992). Tumour necrosis factor α (TNF- α) is crucial for NK recruitment to the TME and mice lacking TNF- α have defective tumor immunosurveillance (Smyth *et al.*, 1998). Additionally, M1 macrophages produce chemokines CXCL9, CXCL10 and CXCL11 that attract more anti-tumorigenic cells to the TME (Haabeth, Bogen and Corthay, 2012).

Cytokines participate in the conversion from tumor-inhibitory to tumor-promoting TME. The balance shifted towards anti-inflammatory cytokines rather than pro-inflammatory cytokines creates an immunosuppressive milieu that allows escape from tumor immunosurveillance (Burkholder *et al.*, 2014). Interleukin-10 and TGF- β produced by different cell populations are the two major regulators of immunosuppression in the TME (Figure 1.6) (Kim *et al.*, 2006).

Interleukin-10 is an immunosuppressive cytokine that allows escape of tumor cells from immune destruction in the TME (Mannino *et al.*, 2015). High expression of IL-10 is considered a poor prognostic factor for patients with melanoma (Dummer *et al.*, 1995; Nemunaitis *et al.*, 2001). Type 2 lymphocytes (T_H2) seem to be the main source of IL-10 in the TME, but other cells types including Tregs, TAMs, DC, NK and tumors cells also produce IL-10. The major mechanism of IL-10 immunosuppression is the impairment of antigen presentation (Sato *et al.*, 2011). Interleukin-10 downregulates co-stimulatory (B7) (Ding *et al.*, 1993) and MHC molecules of APCs (de Waal Malefyt *et al.*, 1991). Interleukin-10 also induces immunosuppressive activity of Tregs and suppresses pro-inflammatory T_H17 cells (Oft, 2014). Further, *in vitro* studies have shown that IL-10 turns CTLs

unresponsive to antigens (Groux *et al.*, 1998) and inhibits the production of IFN- γ cytokine in T_H1 (Taga and Tosato, 1992) and NK cells (Peppas *et al.*, 2010). Some studies have challenged the paradigm that IL-10 exclusively supports tumor development. In some contexts, IL-10 may play anti-tumorigenic roles by increasing the proliferation of CTLs (Chen and Zlotnik, 1991) and NK cell cytotoxic activity (Parato *et al.*, 2002).

Similar to the ambiguous role of IL-10, the role of immunosuppressive cytokine TGF- β in tumor development is also paradoxical and context-dependent. Early in tumorigenesis TGF- β prevents tumor formation by supporting apoptosis and inhibiting cell cycle progression. During late stages of tumor formation TGF- β promotes invasion and immunosuppression (Landskron *et al.*, 2014). Many cell populations secrete TGF- β in the TME including the cancer cells, macrophages, MDSCs, lymphocytes, fibroblasts and endothelial cells (Massagué, 2008). Overall, TGF- β in the TME suppresses the development, proliferation, and function of anti-tumorigenic cells (Pickup, Novitskiy and Moses, 2013). Mice with defects in the TGF- β signaling pathway develop benign neoplasia associated with inflammation that eventually evolves to malignant tumors (Engle *et al.*, 2002). The most notable feature of TGF- β in the TME is to polarize macrophages towards M2 phenotype (Gong *et al.*, 2012). The suppression of T_H1 response and T cell proliferation is also mediated by TGF- β signaling (Gorelik, Constant and Flavell, 2002). Transforming growth factor β inhibits the antitumor response of CTLs (Thomas and Massagué, 2005) as well as impairs the maturation of NK (Marcoe *et al.*, 2012) and DCs, which otherwise would be able to detect cancer cells and initiate the

tumor clearance process (Novitskiy *et al.*, 2012). Lastly, TGF- β induces the differentiation of naïve CD4⁺ cells into Treg, a very immunosuppressive subtype that in addition to suppression of immune response also produces TGF- β (Chen *et al.*, 2003).

Chemokines constitute a subgroup of cytokines responsible for the recruitment of immune cells along the concentration gradient of a ligand, a process known as chemotaxis. Chemokine receptors belong to the G-protein coupled receptor (GPCR) family of receptors and bind to more than one type chemokine ligand. Chemokines are divided into four groups (CXC, CC, C, CX3C) determined by the position of the first two cysteine residues in their protein sequence (Balkwill, 2004). The trafficking of immune cells to the TME is mediated by combinations of different chemokines. Hence, chemokines are considered important regulators of anti-tumor immunity and immune detection escape (Amedei, Prisco and M. D'Elis, 2013). Tumor-inhibitory cells CTLs, NK and T_H1 cells express the chemokine receptor CXCR3 and are intensively recruited to the TME in response to chemokine ligands CXCL9, CXCL10, and CXCL11 (Nagarsheth, Wicha and Zou, 2017). The DCs expressing CCR6 migrate to the TME in response to CCL20 produced by tumor cells (Bell *et al.*, 1999). Recruitment of macrophages to tumors is mainly governed by CCR2-CCL2 (Qian *et al.*, 2011) and CCR5-CCL5 chemokine axes (Azenshtein *et al.*, 2002). Regulatory T cells expressing CCR4 are recruited to the TME by CCL22 derived from tumor cells and macrophages (Curiel *et al.*, 2004). The main chemokines attracting M-MDSCs to the tumors are

CCL2 and CCL5. PMN-MDSCs rely on chemokines CXCL5, CXCL6 and CXCL8 to find their way to the TME (Kumar *et al.*, 2016).

Recently, immunotherapy approaches targeting only a single chemokine axis have failed to produce satisfactory results in patients with chronic inflammatory diseases. The therapeutic failure probably happened as a result of the pleiotropic effect of chemokines. In the context of cancer, targeting a specific chemokine axis is most certainly affecting different population of cells in the TME. Thus, combinatorial strategies aiming to simultaneously stimulate anti-tumor and silence pro-tumor chemokines axes could potentially achieve meaningful results (Nagarsheth, Wicha and Zou, 2017).

1.2.3. Melanoma Microenvironment

The strong interaction between tumorigenic cells and all other cell types in the TME sustain melanoma development (Passarelli *et al.*, 2017). At normal conditions the proliferation of melanocytes is firmly regulated by keratinocytes through the expression of adhesion molecules. During tumorigenesis melanoma cells circumvent the microenvironmental regulation of proliferation by downregulating E-cadherin and overexpressing N-cadherin. The expression of N-cadherin also mediates the interaction of melanoma cells with fibroblasts and endothelial cells in the TME (Villanueva and Herlyn, 2008). A paracrine loop relying on growth factors supports the proliferation of both melanoma cells and fibroblasts in the TME. Melanoma cells secrete platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) that stimulate fibroblasts to proliferate and to secrete growth

factors insulin-like growth factor 1 (IGF-1) and HGF, further supporting melanoma cell proliferation (Melnikova and Bar-Eli, 2009).

Melanoma has long been recognized as one of the most immunogenic types of tumor, having the capacity to elicit strong anti-tumor responses (Passarelli *et al.*, 2017). Melanoma associated antigens such as melan-a, gp100, and tyrosinase can be detected by T cells and trigger tumor-specific immune responses (Castelli *et al.*, 2000). The UV-induced high somatic mutation burden in melanoma creates TAA that can be detected by immune cells (Wang *et al.*, 2017). As such, melanomas with high mutation load have good response to immunotherapies (Ejeta *et al.*, 2015). Recent findings have suggested that melanoma arises in a chronic inflammatory environment (Umansky and Sevko, 2012). Oncogenic signals are important not only for tumor cells' deregulated proliferation but also for creating an inflammatory milieu. Forced expression of BRAF^{V600E} in normal melanocytes results in the production of IL-1 and IL-6 (Kuilman *et al.*, 2008). Expression of pro-inflammatory cytokines IL-1, IL-6, and TNF- α are upregulated when nevi are compared to thick primary melanomas (Moretti *et al.*, 1999). Additionally, melanoma cells express a variety of chemokines ligands including CCL2, CCL5, CXCL1-3, CXCL5-8, and CXCL10 that recruit different populations of immune cells to the TME (Richmond, Yang and Su, 2009).

Although remarkably immunogenic, melanoma develops a variety of strategies to circumvent immune detection. Melanoma cells downregulate MHC-I molecules impairing cytotoxic T cell activity (Ferrone and Marincola, 1995). Melanoma cells

do not display the ligands for NK cell activating receptors on their surface, allowing them to escape NK destruction (Fuertes *et al.*, 2008). Melanoma cells express PD-L1, the ligand to the immune checkpoint PD-1, suppressing melanoma-specific CTLs (Yang *et al.*, 2008). The expression level of PD-L1 in melanoma tumors is positively correlated with aggressiveness (Hino *et al.*, 2010). The melanoma genotype is also associated with the impairment of anti-tumor immune response in the TME (Binnewies *et al.*, 2018). In addition to inflammatory cytokines, BRAF^{V600E} expression in melanoma cells drives the production of immunosuppressive cytokine IL-10 contributing to the evasion of immune detection (Sumimoto *et al.*, 2006). Melanocytic expression of BRAF^{V600E} and β -catenin coupled with *Pten* deletion in a mouse model generated aggressive melanomas that were poorly infiltrated by CTLs because of the lack of DCs in the TME. The expression of β -catenin in melanoma cells reduces the availability of CCL4 chemokines, which in turn diminishes the trafficking of DCs to the microenvironment (Spranger, Bao and Gajewski, 2015).

Undoubtedly, immune cells are central players of immunosuppression in the melanoma microenvironment. The seminal work by Tirosh and colleagues expanded our understanding about the immune cells in the melanoma TME. The authors performed single-cell RNA sequencing (RNA-Seq) of 4645 malignant and non-malignant cells from 19 patients with metastatic melanoma to establish the profile of stromal cells in the melanoma TME. They showed that the melanoma ecosystem is consisted mainly of T (CD8⁺, CD4⁺ and Treg) and B lymphocytes. In addition, there are other immune populations, namely macrophages and NK cells

and stromal cells including CAFs and endothelial cells. In the T cell infiltrate of metastatic melanomas, CTLs express cytotoxic genes together with exhaustion markers PD-1 and CTLA-4. Furthermore, clonal expanded CTLs show signs of exhaustion indicating the suppression of immune surveillance. Tregs represent a small population of lymphocytes (57 out of 2068 cells) in TME. These immunosuppressive cells overexpress immune checkpoint CTLA-4 and have a moderate to low expression of cytotoxic marker GZB (Tirosh *et al.*, 2016). Transforming growth factor β is central to the Tregs mediated immunosuppression in the melanoma TME. In addition to inhibiting cytotoxic T cell functions and APCs, TGF- β also promotes Treg proliferation (Braeuer *et al.*, 2014). Accordingly, these data strongly suggest that melanoma is a type of “cold” tumor, thus in the melanoma TME there are few activated CTLs and many immunosuppressive cells preventing anti-tumor cytotoxic activity (Binnewies *et al.*, 2018).

The intense research on melanoma microenvironment has paved the way for the development of immunotherapies that have recently revolutionized the management of melanoma disease producing long-lasting successful responses (Giavina-Bianchi, Giavina-Bianchi Junior and Festa Neto, 2017). Melanoma immunotherapy has been particularly focused on the employment of immune checkpoint inhibitors anti-CTLA-4 and anti-PD-1, in order to rescue activation and function of tumor-specific lymphocytes, respectively (Binnewies *et al.*, 2018). Cytotoxic T lymphocyte associated protein 4 (CTLA-4) is an inhibitory molecule expressed by activated T cells that competes with co-stimulatory receptor CD28 for the B7 ligand expressed on the surface of DCs. CTLA-4 normally works as a

molecular brake during the priming of T cells preventing excessive T cell responses (Postow, Callahan and Wolchok, 2015). Preclinical studies blocking CTLA-4 generated effective response against tumors (Leach, Krummel and Allison, 1996) and encouraged the development of clinical trials using human CTLA-4 blocking antibody (ipilimumab). Although preliminary studies had shown that ipilimumab might have an initial delayed response associated with disease progression (Saenger and Wolchok, 2008), CTLA-4 blockage in patients with metastatic melanoma have produced an astonishing durable response for up to a decade (Schadendorf, Hodi, *et al.*, 2015). Ipilimumab was found to be more effective than conventional chemotherapy in the treatment of patients with advanced melanomas (Robert *et al.*, 2011).

Targeting the PD-1 pathway using nivolumab or pembrolizumab blocking antibodies is another very effective immunotherapy approach that has achieved long-term satisfactory results in patients with metastatic melanoma (Topalian *et al.*, 2014). Programed cell death-1 (PD-1) is a negative regulator expressed by exhausted CTLs during chronic immune response in the TME. Binding of PD-L1 expressed on the surface of tumor cells to the PD-1 receptor present in CTLs inactivates immune responses against tumors (Ribas and Wolchok, 2018). A possible explanation for the successful blockage of PD-1 pathway is the presence of infiltrated tumor-specific CTLs in the TME (Haanen, 2017). An effective strategy to convert “cold” melanomas into highly inflamed tumors is through intratumoral injection of oncolytic virus. This procedure induced CTLs infiltration into the TME and, after the treatment with pembrolizumab, resulted in reduction of tumor burden

in more than 60% of patients with metastatic melanoma (Ribas *et al.*, 2017). The immune checkpoints CTLA-4 and PD-1 have temporal and spatial distinct mechanisms of action. Cytotoxic T lymphocyte associated protein 4 regulates the initial steps of immune response in the draining lymph node whereas PD-1 controls the effector phase of immune response in the TME. These observations suggest that combination of CTLA-4 and PD-1 immune checkpoints inhibitors would have synergetic effects in the immune response against tumors (Postow, Callahan and Wolchok, 2015; Ribas and Wolchok, 2018). In fact, melanoma patients treated with pembrolizumab in combination with ipilimumab presented considerable longer durable response rate when compared to monotherapy (Larkin *et al.*, 2015).

In the last few years, a great body of data have shown that the gut microbiota is an important modulator of melanoma response to immunotherapy and can be manipulated to provide better clinical responses (Vancheswaran Gopalakrishnan *et al.*, 2018). Anti-CTLA-4 treatment changed the diversity of microbiota in mice, specifically decreasing the amount of *Bacteroidales* and increasing *Clostridiales* load in the gut. Germ-free mice treated with anti-CTLA-4 did not exhibit anti-tumor response, which was reestablished when mice received immune checkpoint inhibitor in combination with *Bacteroidales* isolates. Furthermore, fecal microbiota transplantation (FMT) from melanoma patients with high amounts of *Bacteroidales* enhanced the anti-tumor effect of CTLA-4 blockage in mice (Vetizou *et al.*, 2015). Interestingly, mice from different vendors but with the same genetic background responded differently to PD-1 pathway blockage. The determinant of the distinct response was the amount of *Bifidobacterium* in the microbiota. Mice with more

Bifidobacterium in the gut showed delayed melanoma growth when treated with anti-PDL-1. When mice with low amounts of *Bifidobacterium* were treated with these bacteria they presented improved anti-tumor response to PD-L1 blockage. *Bifidobacterium* positively influences DCs function and consequently improves anti-tumor activity of CTLs (Sivan *et al.*, 2015). Recently, these findings were shown to have clinical relevance for patients with metastatic melanoma (Jobin, 2018). As observed in the preclinical studies, melanoma patients responding (R) to PD-1 blockage had distinct microbiota profile compared to those not responding (NR) to treatment. Favorable microbiota augmented the number of APCs and CTLs in the TME as well as the frequency of CTLs compared to Tregs. Mouse models validated these observations and confirmed that FMT from R melanoma patients to germ-free mice was able to improve anti-tumor response to anti-PD-1 treatment (Matson *et al.*, 2018; V. Gopalakrishnan *et al.*, 2018).

1.3. Endothelin

Endothelin (Edn) is a cytokine ubiquitously expressed in various organs and has remarkable pleiotropic functions. There are 3 isoforms of endothelins - Edn1, Edn2, and Edn3 - that bind to 2 different GPCRs, endothelin receptor type A (Ednra) and type B (Ednrb). Endothelin 1 is the predominant and more relevant isoform and binds with the same affinity to both Ednra and Ednrb. Edn2 also has similar affinity to Ednra and Ednrb whereas Edn3 is very selective for Ednrb (Aubert and Juillerat-Jeanneret, 2016). Binding of endothelin to one of its cognate receptors triggers the stimulation of a variety of downstream effectors (Rosanò, Spinella and Bagnato, 2013). Activation of endothelin receptor initiates PLC β

signaling pathway resulting in PKC activation (Simonson and Dunn, 1990). Endothelin receptors activate MAPK and PI3K pathways leading to ERK phosphorylation (Iwata *et al.*, 2009) and mTOR activation (Wu *et al.*, 2012). Different cell types secrete endothelins including endothelial cells, cardiomyocytes, leukocytes, glial cells and epithelial cells in the lung and kidney. Equally diverse is the roster of cell populations that express receptors to endothelin and includes among others, endothelial and smooth cells, neurons, hepatocytes, adipocytes, keratinocytes, and melanocytes (Khimji and Rockey, 2010). Of note, the expression pattern of each endothelin varies regarding organs and cell types (Masaki, 2004).

1.3.1. Endothelin Physiology and Pathology

Endothelins were first discovered as mediators of vascular function (Yanagisawa *et al.*, 1988). Endothelial cells secrete Edn1 that binds to the Ednra expressed on smooth cells and trigger vasoconstriction. Endothelin 1 is considered to be the most potent vasoconstrictor already described. Endothelin 1 derived from endothelial cells can also bind to Ednrb expressed by neighboring endothelial cells. Stimulation of Ednrb in endothelial cells induces the secretion of nitric oxide (NO), a strong vasodilator for smooth cells (Kawanabe and Nauli, 2011). Endothelins have many other physiological functions besides vasoconstriction. For example, endothelins regulate development of neural crest cells (Kedzierski and Yanagisawa, 2001) bronchoconstriction in the lungs (Uchida *et al.*, 1988) and water and salt excretion in the kidneys (Kohan, 2006). Shortly after endothelin discovery, a variety of pathological conditions were found to be associated with

alterations in endothelin physiology. Cardiovascular diseases including hypertension and atherosclerosis, pulmonary arterial hypertension and glomerulosclerosis are all diseases that have an inflammatory component associated with deregulation in endothelin signaling suggesting that endothelin modulates immune system functions (Barton and Yanagisawa, 2008).

The inflammatory process involves changes in the vascular permeability and trafficking of leukocytes to the sites of tissue injury. Endothelin is associated with these events and therefore, is considered a pro-inflammatory cytokine (Figure 1.7) (Nett *et al.*, 2006). The transcription factor NF- κ B, a central regulator of inflammation, is under the control of endothelin signaling (Wilson, Simari and Lerman, 2001). Endothelin increases local vascular permeability (Filep *et al.*, 1992) and stimulates the expression of adhesion molecules (i.e., ICAM-1) on endothelial cells (Hayasaki *et al.*, 1996), allowing the infiltration of leukocytes into the inflammation site. Endothelin in the inflammatory milieu stimulates neutrophil accumulation (López Farré *et al.*, 1993) and is a chemoattractant for monocytes (Hanggono Achmad and Rao, 1992), and macrophages. Of note, the chemical structure of mature endothelins resembles that of some chemokines (Grimshaw, Wilson and Balkwill, 2002). Secretion of pro-inflammatory cytokines IL-1, IL-6, TNF- α , and IFN- γ is regulated by endothelin expression (Yang *et al.*, 2004). Interestingly, activated T cells produce TNF- α and IFN- γ inducing monocytes to secrete endothelin, which in turn enhances the inflammatory process (Shinagawa *et al.*, 2017). In addition to endothelial cells and monocytes, DCs (Guruli *et al.*,

2004) and macrophages (Ehrenreich *et al.*, 1990) are other sources of endothelin in the inflammatory environment.

1.3.2. Endothelin signaling in melanocytes and melanoma cells

Many cytokines are associated with melanocyte and melanoma biology, among them are the endothelins. The *Edn3/Ednrb* pathway has been implicated in melanocyte development as well as melanoma progression (Saldana-Caboverde and Kos, 2010). Endothelin 3 regulates the proliferation and survival of melanocyte precursors (Lahav *et al.*, 1996, 1998) and promotes melanoblast differentiation into melanocytes (Reid *et al.*, 1996). The essential role of endothelin signaling during melanocyte development is demonstrated by mouse models with spontaneous mutations in *Edn3* (Baynash *et al.*, 1994) and *Ednrb* genes (Hosoda *et al.*, 1994). These mice have hypopigmented phenotypes because of the reduction in the number of melanoblasts and impaired migration of melanocyte precursors during development (Pavan and Tilghman, 1994; Lee, Levorse and Shin, 2003). Conversely, overexpression of *Edn3* in the mouse skin leads to an augmented melanoblast population and a hyperpigmented phenotype (Garcia *et al.*, 2008). The communication between keratinocytes and melanocytes is mediated by endothelin. Upon UV light exposure, keratinocytes release *Edn1* (Imokawas, Yada and Miyagishi, 1992) that stimulates melanin production in the melanocytes (Imokawa *et al.*, 1997). Thus, endothelin may have an indirect protective effect against melanoma initiation (Saldana-Caboverde and Kos, 2010).

Initially, EDNRB was thought to be expressed at low levels in melanoma cells. Profiling of a cDNA library detected the downregulation of EDNRB in 16 out of 17 human melanoma cells lines (Eberle *et al.*, 1999). Analysis of primary and metastatic melanoma cell lines reported that EDNRB is downregulated when metastatic cells are compared to primary melanoma (Kikuchi *et al.*, 1996). Further studies including human samples challenged the *in vitro* findings and showed that EDNRB expression increases during melanoma progression. A gene profiling study of melanoma cell lines and tumor biopsies showed that EDNRB is overexpressed in most of human melanomas (Bittner *et al.*, 2000). Demunter *et al.* (2001) demonstrated that EDNRB expression gradually increases from benign nevi to metastatic melanoma and proposed EDNRB as marker for melanoma progression (Demunter *et al.*, 2001). Tang *et al.* (2008) were the first to address the role of EDN3 on melanoma progression. The analysis of biopsies and cell lines revealed that metastatic melanoma cells express high levels of EDN3 suggesting that its autocrine secretion might modulate melanoma metastasis (Tang *et al.*, 2008). Using human samples and a mouse model of melanoma brain metastasis, Cruz-Munoz *et al.* (2012) showed that the overexpression of EDNRB promotes melanoma metastatic growth within the central nervous system (CNS) (Cruz-Muñoz *et al.*, 2012). The proliferation of metastatic melanoma cells in the brain might take place as a result of EDNRB interaction with EDN3 since this ligand is abundant in the CNS (Davenport *et al.*, 2016). Additional studies demonstrated that Ednrb activation triggers many molecular events relevant for melanoma metastasis. Binding of EDN1 or EDN3 to EDNRB downregulates E-cadherin

(Jamal and Schneider, 2002) and increases the expression of N-cadherin and as well as secretion of MMPs. Endothelin Receptor B activation also stimulates proliferation, migration, and invasion of melanoma cells (Bagnato *et al.*, 2004). Altogether, these findings strongly suggest that the Ednrb/Edn3 axis contributes significantly to melanoma progression. Figure 1.8 summarizes the effects of endothelin signaling in melanoma cells.

As discussed above a well-established body of data establishes that EDNRB plays tumor-promoting roles during melanoma development. Conversely, few studies have addressed the tumor suppressive aspects of EDNRB. These reports suggest that, in some specific contexts, EDNRB can act as a tumor suppressor gene preventing melanoma initiation. In the RET transgenic mouse model of melanoma, Ednrb expression is reduced when malignant tumors are compared to benign melanocytic lesions. Furthermore, RET transgenic mice carrying a heterozygous deletion of Ednrb (Ednrb^{+/-}; RET-mouse) develops *de novo* melanomas that are more prone to metastasize to the lungs compared to RET-mouse (Kumasaka *et al.*, 2010). Additional studies using the same mouse model revealed that the reduction of Ednrb levels is associated with Plexin downregulation. Plexin is considered a melanoma tumor suppressor (Scott *et al.*, 2009) and the positive correlation between Ednrb and Plexin expression supports a possible tumor suppressive role of Ednrb (Kumasaka *et al.*, 2015). Variants in the EDNRB gene might underscore the tumor suppressive effect of EDNRB. Berger *et al.* (2006) demonstrated that EDN1 had reduced binding to an EDNRB variant found in a human melanoma cell line. This EDNRB variant is probably

defective in the N- and C- terminal domains making the receptor unable to bind to endothelins (Berger, Bernasconi and Juillerat-Jeanneret, 2006).

The relevance of EDNRB variants for melanoma incidence is still under debate. Caucasians harboring the germline variant S305N in the EDNRB gene are associated with increased predisposition to melanoma (Soufir *et al.*, 2005). However, two case-control studies, one in Germany (Thirumaran *et al.*, 2006) and another in Spain (Fernandez *et al.*, 2009) claimed that S305N variant is not associated with melanoma risk. The Spanish study took in consideration only sporadic melanoma cases. Still, some reports have linked EDNRB mutation with familial melanoma. A study with 2 independent large populations of Caucasians showed that S305N variant in EDNRB gene is not linked with sporadic melanoma but is significantly associated with CDKN2A mutations, which is responsible for familial melanoma. Therefore EDNRB variants might be important players of inheritably susceptibility to melanoma (Spica *et al.*, 2011).

1.3.3. EDNRB and melanoma: clinical opportunities

Notwithstanding the recent advances regarding targeted therapy and immunotherapy, melanoma treatment still faces many challenges. Clinical targeting of EDNRB emerges as a very promising approach despite the paradoxical findings. The most explored strategy is to block EDNRB using bosentan, a dual antagonist to EDNRA/EDNRB, or antagonist selective for EDNRB such as BQ788, A-192621. Some of these inhibitors are routinely employed for treating cardiovascular or pulmonary dysfunctions and their therapeutic features

have been assessed in the context of melanoma (Aubert and Juillerat-Jeanneret, 2016). Most of the data exploring blockage of endothelin signaling in melanoma came from studies using cell lines and preclinical mouse models (Lahav, Heffner and Patterson, 1999; Rosanò *et al.*, 2004; Smith *et al.*, 2017). Clinical trials aiming to block EDNRB in patients with melanoma demonstrated limited beneficial results and therefore might require additional investigation (Kefford *et al.*, 2007; Wouters *et al.*, 2015).

The endothelin receptor inhibitor BQ-788 very efficiently competes with EDN1 for EDNRB, but not EDNRA, and is considered the strongest EDNRB antagonist (Ishikawa *et al.*, 1994). Melanoma cell lines exposed to BQ-788 showed cell growth inhibition as well as enhanced cell death. The same effects were not observed when melanoma cells are treated with EDNRA selective inhibitor. Furthermore, BQ788 treatment abrogated tumor growth in melanoma xenografts (Lahav, Heffner and Patterson, 1999). The apoptotic effect of BQ-788 was more prominent in aggressive melanoma cells and relied on the reduction of survival factor BCL2A1 expression associated with caspase-6 activation (Lahav *et al.*, 2004). Bagnato and colleagues extensively explored the role of EDNRB blockage in melanoma. They showed that BQ-788 impairs the phosphorylation of ERK and decreases melanoma cell proliferation. BQ788 treatment also prevents upregulation of N-cadherin, downregulation of E-cadherin as well as secretion of metalloproteinases (Bagnato *et al.*, 2004).

Modifications on the chemical structure of EDNRA antagonists led to the development of the oral non-peptide selective EDNRB antagonist A192621 (von Geldern *et al.*, 1999). Administration of A192621 to melanoma xenografts had remarkable tumor suppressor effects. The EDNRB antagonist A192621 abolished tumor cell proliferation as well as reduced tumor volume in nude mice (Bagnato *et al.*, 2004; Spinella *et al.*, 2007). Melanoma brain metastatic cell lines exhibited decreased cell proliferation when treated with A192621. Monotherapy with A192621 is ineffective in brain metastasis treatment because of the inability of A192621 to cross the blood-brain barrier (BBB). However, when A192621 treatment was associated with inhibitors of efflux pump P-glycoprotein (PGP), the EDNRB inhibitor was able to penetrate the CNS and decrease the size of melanoma brain metastasis (Cruz-Muñoz *et al.*, 2012).

Tumorigenesis is highly dependent on the process of angiogenesis. Therapeutic inhibition of EDNRB has controversial effects in melanoma angiogenesis. The treatment with BQ-788 was shown to play a pro-angiogenic role. Melanoma cells treated with BQ-788 showed increased expression of VEGF and hypoxia-inducible factor-1 α (HIF1 α) and downregulation of the angiogenic suppressor *gravin*. It is possible that the increased expression of VEGF resulting from EDNRB blockage encourages endothelial cells to proliferate (Lahav *et al.*, 2004). Patients treated with BQ-788 exhibited abundance of blood vessels and increased expression of HIF1 α . Although angiogenesis is helpful for melanomas as it increases the blood supply, the formation of new blood vessels can also be harmful to tumors since they create new routes for immune cells to be recruited to

the TME (Wouters *et al.*, 2015). Conversely, A192621 treatment seems to have evident anti-angiogenic effect in melanoma. Melanoma xenografts treated with A192621 presented increased PDH2 expression and downregulation of HIF1 α , this in turn impaired angiogenesis and tumor growth (Spinella *et al.*, 2007, 2010).

Despite the successful anti-melanoma effect of EDNRB blockage in preclinical studies, the clinical relevance of this approach is still elusive. Bosentan is a non-peptide dual EDNRA and EDNRB antagonist. Preclinical studies showed that this EDNRB inhibitor reduces cell viability and stimulates apoptosis in human melanoma cells. The apoptotic effect of bosentan is enhanced when it is combined with chemotherapy drug dacarbazine (DTIC) (Berger, Bernasconi and Juillerat-Jeanneret, 2006). These results encouraged clinical trials that addressed the anti-tumor effect of bosentan in patients with metastatic melanoma. A phase II clinical study showed that 6 out 32 patients with stage IV melanoma presented disease stabilization upon monotherapy with bosentan (Kefford *et al.*, 2007). Unfortunately, in a supplementary study, the combination of bosentan with DTIC showed no effect in tumor progression and survival of patients with metastatic melanoma (Kefford *et al.*, 2010). One of the potential reasons for the unsuccessful outcomes of EDNRB blockage in melanoma patients is the absence of previous screening for patients with high levels of intratumoral EDNRB. Blocking EDNRB using BQ788 seems to be more effective for melanoma patients. A recent clinical study demonstrated that intralesional administration of the selective EDNRB antagonist directly reduced the expression of the proliferation marker Ki-67 as well as diminished tumor growth. Treatment with BQ-788 also enhanced immune cell

infiltration in melanoma, suggesting that future studies evaluating the efficacy of a combination of EDNRB inhibitor and immune checkpoint inhibitors should be carried out (Wouters *et al.*, 2015).

To overcome the low sensitivity of small molecule antagonists such as BQ-788, functional monoclonal antibodies (mAb) can also be used as therapeutic strategies. Rendomab-B1 was the first described mAb with antagonist ability to the human EDNRB. Rendomab-B1 exhibits dual pharmacological action, it competes with EDN1 ligand as well as promotes EDNRB internalization (Allard *et al.*, 2013). Another approach that has been tested in melanoma is antibody-drug conjugated (ADC) therapy that combines antibodies with potent cytotoxic agents. Asundi and colleagues (2011) developed a monoclonal antibody (5E9) conjugated with the potent cytotoxic compound MMAE. The ADC 5E9 has high affinity to EDNRB and is rapidly internalized. Antibody-drug conjugated 5E9 shows remarkable anti-tumor activity in both melanoma cell lines and xenograft models expressing EDNRB (Asundi *et al.*, 2011). Further studies assessed the combinatorial effect of anti-EDNRB ADC with MAPK inhibitors. The exposure to MAPK inhibitors to cell lines and tumor models harboring either wild type or mutant BRAF and NRAS promoted expression of EDNRB, even in cells resistant to MAPK inhibitors. The increased expression of EDNRB in turn enhanced the anti-tumor activity of the ADC. Taken together, these data indicate that the combination of MAPK inhibitors with anti-EDNRB ADC might be a powerful and effective approach in a broad range of patients with melanoma (Asundi *et al.*, 2014).

A more recent study explored the role of endothelin during resistance to BRAFi. Treatment with BRAFi increases MITF expression in melanoma cells, which is heterogeneously maintained among tumors cells. When MITF^{high} and MITF^{low} melanoma cells were co-cultured in the presence of BRAFi, MITF^{high} protected MITF^{low} melanoma cells and stimulated their growth. The shielding effect was maintained by EDN1 released from BRAFi resistant cells which protected otherwise drug-sensitive melanoma cells and led to ERK re-activation via PCK and CRAF pathways activation. Endothelin signaling also perturbs AXL^{high} melanoma cells since monotherapy with BRAFi increased AXL levels and combination with endothelin blockage reduced AXL expression. These findings suggest the endothelin signaling is an important regulator of melanoma resistance to BRAFi (Smith *et al.*, 2017).

Furthermore, endothelin coordinates the phenotypic heterogeneity that takes place when tumors become resistant to BRAFi. The phenotypic heterogeneity is demonstrated by the correlation between endothelin receptors and MITF/AXL expression levels. Melanoma cells with MITF^{high} phenotype have an increased expression of EDNRB but reduced levels of EDNRA; in AXL^{high} tumor cells this correlation is inverted. Therefore, BRAFi treatment increases MITF expression which in turn leads to EDN1 production. Endothelin 1 might have an autocrine effect and binds to EDNRB in MITF^{high} cells (or paracrine in other MITF^{high} cells) re-activating ERK and consequently regulating proliferation and survival. Endothelin 1 might also act in a paracrine fashion in AXL^{high} cells upregulating EDNRA. In the paracrine context, endothelin signaling re-activates ERK but

promotes only cell proliferation. In addition to interfering with the activation of BRAF, combination of BRAFi with endothelin receptor blockage might also act in the TME as endothelin is crucial for many stromal cells (Smith *et al.*, 2017).

1.4. Research Questions

Melanoma is one of the most immunogenic types of tumors and is also known to grow in an immunosuppressive environment. The latter affords tumorigenic cells with the ability to escape the immune system and ultimately progress and metastasize. The Edn3/Ednrb pathway is considered a marker for melanoma progression. Still, it is not clear whether melanoma cells themselves or other cells present in the TME respond to endothelin signaling. Despite its many demonstrated pro-tumorigenic functions, it is still uncertain if endothelin is important for immune escape. Regulatory T cells are strong immunosuppressive cells present in the TME that limit tumor-specific immune responses and promote immune evasion as well as cancer progression. Currently there is no information about Treg cells responding to endothelin signaling in melanoma. My central hypothesis is that **overexpression of Edn3 creates an immunosuppressive environment promoting melanoma growth and progression**. To test the hypothesis, I will address the following questions:

Question 1: Does Edn3/Ednrb signaling promote melanoma progression?

I evaluated the role of Edn3 signaling in melanoma progression by injecting relevant murine melanoma cell lines in an immunocompetent mouse model that overexpresses Edn3 in the skin (*K5-tTA;TRE-Edn3-lacZ* which will be referred to

as *K5-Edn3*). I found that when Edn3 is overexpressed in the TME, tumors grow significantly more and are more aggressive.

Question 2: Can tumorigenic and stromal cells respond to Edn3 in the microenvironment?

To address this question I characterized Ednrb expression in different population of immune cells present in the melanoma TME. I observed that Treg is the only population in TME that has consistently different amounts when Edn3 is overexpressed.

Question 3: Does Edn3/Ednrb signaling play immunosuppressive roles in the melanoma TME?

I addressed the inhibitory effect of endothelin signaling during the immune response against melanoma. I found that Endothelin was required for immune escape of highly immunogenic melanoma cells. The immunosuppressive features of Tregs were enhanced in presence of Edn3. Overexpression of Edn3 in the melanoma microenvironment resulted in upregulation of immunosuppressive cytokines and decreased levels of cytokines that elicit anti-tumor immunity. Melanomas exposed to high levels of Edn3 were sensitive to immune checkpoint inhibitor as well as to Ednrb blockage.

Figure 1.1 Melanoma progression pathways

The linear pathway of progression from melanocytes to non-CSD or CSD metastatic melanomas involves the development of intermediate lesions through the acquirement of a series of genetic alterations. Alternatively, during the parallel pathway the development of melanocytic lesions may not require some precursor lesions to form metastatic melanoma.

Figure 1.2 Cells types and signaling molecules in the tumor microenvironment (TME)

The different cell populations present in the tumor microenvironment and their relationship is represented here. Cells from the tumor immune surveillance system can detect nascent cancer cells and eliminate them. Immune suppressive cells impair the mechanisms of immune surveillance which favor tumor progression. CAFs, endothelial cells and other signaling molecules also support the tumor development.

Figure 1.3 Macrophage polarization

Monocytes can be polarized towards M1 or M2 type of macrophages. The cytokine IFN- γ polarizes monocytes towards M1 phenotype, which produces IL-1, IL-6, IL-12 and TNF- α cytokines. M2 macrophages are induced by IL-10, TGF- β , IL-4 and IL-13 cytokines and produce anti-inflammatory cytokines.

Figure 1.4 Naïve CD4⁺ T cell differentiation

Naïve CD4⁺ T cell differentiates into different effector subtypes depending on which cytokines are available during antigen presentation by APCs. Each subtype has a different biological role and is distinguished by its own cytokine profile.

Figure 1.5 Tumor inhibitory cytokine network

A small number of cytokines are responsible for controlling tumor immune surveillance. T_H1 cells secrete IL-2 (which sustain the proliferation of cytotoxic cells) and IFN-γ, a cytokine that polarizes macrophages towards M1 phenotype. M1 macrophages produce IL-12 and pro-inflammatory cytokines IL-1, IL-6 and TNF-α that regulate anti-tumor immunity.

Figure 1.6 Cellular targets of immunosuppressive cytokines

IL-10 and TGF- β are central modulators of immunosuppression in the TME. These cytokines inhibit the anti-tumor activity of NK cells, T_H1, CTL, DC and M1 cells and activate Treg cells.

Figure 1.7 Endothelin and inflammation

Endothelin regulates important events that happen during inflammation. Endothelin increases vascular permeability and the expression of adhesion molecules in blood vessels. Endothelin also recruits leukocytes and stimulates the secretion of pro-inflammatory cytokines. Endothelial cells, macrophages, DCs and monocytes are some of the cell populations that supply the inflammatory milieu with endothelin.

Figure 1.8 Endothelin signaling activation in melanoma cells

The activation of Edn3/Ednrb axis in melanoma cells triggers many cellular mechanisms that promote melanoma progression.

1.6. References

- Ackermann, J. *et al.* (2005) 'Metastasizing melanoma formation caused by expression of activated N-RasQ61K on an INK4a-deficient background', *Cancer Research*, 65(10), pp. 4005–4011. doi: 10.1158/0008-5472.CAN-04-2970.
- Ahmed, A. A. *et al.* (1995) 'Interleukin (IL)-1 α - and -1 β -, IL-6-, and Tumor Necrosis Factor- α -like Immunoreactivities in Human Common and Dysplastic Nevocellular Nevi and Malignant Melanoma', *The American Journal of Dermatopathology*, 17(3), pp. 222–229. doi: 10.1097/00000372-199506000-00002.
- Alexandrov, L. B. *et al.* (2013) 'Signatures of mutational processes in human cancer', *Nature*, 500(7463), pp. 415–421. doi: 10.1038/nature12477.
- Allard, B. *et al.* (2013) 'Generation and characterization of rendomab-B1, a monoclonal antibody displaying potent and specific antagonism of the human endothelin B receptor', *MABs*, 5(1), pp. 56–69. doi: 10.4161/mabs.22696\n22696 [pii].
- Allavena, P. *et al.* (2008) 'The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance', *Immunological Reviews*, 222(1), pp. 155–161. doi: 10.1111/j.1600-065X.2008.00607.x.
- Amedei, A., Prisco, D. and M. D'Elios, M. (2013) 'The Use of Cytokines and Chemokines in the Cancer Immunotherapy', *Recent Patents on Anti-Cancer Drug Discovery*, 8(2), pp. 126–142. doi: 10.2174/157489213805290556.
- Andersen, M. H. *et al.* (2006) 'Cytotoxic T cells', *Journal of Investigative Dermatology*. Elsevier Masson SAS, 126(1), pp. 32–41. doi: 10.1038/sj.jid.5700001.
- Appay, V., Douek, D. C. and Price, D. A. (2008) 'CD8+T cell efficacy in vaccination and disease', *Nature Medicine*, 14(6), pp. 623–628. doi: 10.1038/nm.f.1774.
- Aribia, M.-H. B. *et al.* (1987) 'rIL 2-induced proliferation of human circulating NK cells and T lymphocytes: Synergistic effects of IL 1 and IL 2', *Journal of Immunology*, 139(2), pp. 443–451.
- Asundi, J. *et al.* (2011) 'An Antibody-Drug Conjugate Targeting the Endothelin B Receptor for the Treatment of Melanoma', *Clinical Cancer Research*, 17(5), pp. 965–975. doi: 10.1158/1078-0432.CCR-10-2340.
- Asundi, J. *et al.* (2014) 'MAPK pathway inhibition enhances the efficacy of an anti-endothelin B receptor drug conjugate by inducing target expression in melanoma.', *Molecular cancer therapeutics*, 13(6), pp. 1599–1610. doi: 10.1158/1535-7163.MCT-13-0446.

- Aubert, J. D. and Juillerat-Jeanneret, L. (2016) 'Endothelin-Receptor Antagonists beyond Pulmonary Arterial Hypertension: Cancer and Fibrosis', *Journal of Medicinal Chemistry*, 59(18), pp. 8168–8188. doi: 10.1021/acs.jmedchem.5b01781.
- Azenshtein, E. *et al.* (2002) 'The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity', *Cancer Research*, 62(4), pp. 1093–1102.
- Bachmayr-Heyda, A. *et al.* (2013) 'Prognostic impact of tumor infiltrating CD8+ T cells in association with cell proliferation in ovarian cancer patients - a study of the OVCAD consortium', *BMC Cancer*. *BMC Cancer*, 13(1), p. 422. doi: 10.1186/1471-2407-13-422.
- Bagnato, A. *et al.* (2004) 'Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression', *Cancer Res*, 64(4), pp. 1436–1443.
- Balch, C. M. *et al.* (1983) 'A multifactorial analysis of melanoma. III. Prognostic factors in melanoma patients with lymph node metastases (stage II)', *J Clin Oncol*, 1(2), pp. 126–34. doi: 10.1097/00000658-198103000-00023.
- Balch, C. M. *et al.* (2009) 'Final version of 2009 AJCC melanoma staging and classification', *Journal of Clinical Oncology*, 27(36), pp. 6199–6206. doi: 10.1200/JCO.2009.23.4799.
- Balkwill, F. (2004) 'Cancer and the chemokine network', *Nature Reviews Cancer*, 4(7), pp. 540–550. doi: 10.1038/nrc1388.
- Balkwill, F. and Mantovani, A. (2001) 'Inflammation and cancer: Back to Virchow?', *Lancet*, 357(9255), pp. 539–545. doi: 10.1016/S0140-6736(00)04046-0.
- Banchereau, J. and Palucka, A. K. (2005) 'Dendritic cells as therapeutic vaccines against cancer', *Nature Reviews Immunology*, 5(4), pp. 296–306. doi: 10.1038/nri1592.
- Barton, M. and Yanagisawa, M. (2008) 'Endothelin: 20 years from discovery to therapy', *Canadian Journal of Physiology and Pharmacology*, 86(8), pp. 473–484. doi: 10.1139/Y08-058.
- Bautista, N. C., Cohen, S. and Anders, K. H. (1994) 'Benign melanocytic nevus cells in axillary lymph nodes: A prospective incidence and immunohistochemical study with literature review', *American Journal of Clinical Pathology*, 102(1), pp. 102–108. doi: 10.1093/ajcp/102.1.102.
- Baynash, A. G. *et al.* (1994) 'Interaction of Endothelin-3 with Endothelin-B Receptor Is Essential for Development of Epidermal Melanocytes and Enteric Neurons', *Cell*, 79(7), pp. 1277–1285. doi: 10.1016/0092-8674(94)90018-3.

- Beaugerie, L. *et al.* (2013) 'Risk of colorectal high-grade dysplasia and cancer in a prospective observational cohort of patients with inflammatory bowel disease', *Gastroenterology*. Elsevier, Inc, 145(1), p. 166–175.e8. doi: 10.1053/j.gastro.2013.03.044.
- Becker, J. C. *et al.* (2013) 'Immune-suppressive properties of the tumor microenvironment', *Cancer Immunology, Immunotherapy*, 62(7), pp. 1137–1148. doi: 10.1007/s00262-013-1434-6.
- Bell, B. D. *et al.* (1999) 'In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas', *Journal of Experimental Medicine*, 190(10), pp. 1417–26.
- Berger, Y., Bernasconi, C. C. and Juillerat-Jeanneret, L. (2006) 'Targeting the endothelin axis in human melanoma: combination of endothelin receptor antagonism and alkylating agents.', *Experimental biology and medicine*, 231(6), pp. 1111–1119.
- Binnewies, M. *et al.* (2018) 'Understanding the tumor immune microenvironment (TIME) for effective therapy', *Nature Medicine*. Springer US, 24(5), pp. 541–550. doi: 10.1038/s41591-018-0014-x.
- Bittner, M. *et al.* (2000) 'Molecular classification of cutaneous malignant melanoma by gene expression profiling.', *Nature*, 406(6795), pp. 536–540. doi: 10.1038/35020115.
- Braeuer, R. R. *et al.* (2014) 'Why is melanoma so metastatic?', *Pigment Cell & Melanoma Research*, 27(1), pp. 19–36. doi: 10.1111/pcmr.12172.
- Burkholder, B. *et al.* (2014) 'Tumor-induced perturbations of cytokines and immune cell networks', *Biochimica et Biophysica Acta - Reviews on Cancer*. The Authors, 1845(2), pp. 182–201. doi: 10.1016/j.bbcan.2014.01.004.
- Castelli, C. *et al.* (2000) 'T-Cell Recognition of Melanoma-Associated Antigens', *J Cell Physiol*, 183(3), pp. 323–331. doi: 10.1002/(SICI)1097-4652(200003)182:3<323::AID-JCP2>3.0.CO;2-#.
- Chakraborty, A. K. *et al.* (1996) 'Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: Regulation by ultraviolet B', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1313(2), pp. 130–138. doi: 10.1016/0167-4889(96)00063-8.
- Chen, W. *et al.* (2003) 'Conversion of Peripheral CD4+CD25– Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF- β Induction of Transcription Factor Foxp3', *The Journal of Experimental Medicine*, 198(12), pp. 1875–1886. doi: 10.1084/jem.20030152.
- Chen, W. and Zlotnik, A. (1991) 'IL-10: a novel cytotoxic T cell differentiation', *J Immunol*, 147(2), pp. 528–34.

- Clemente, C. G. *et al.* (1996) 'Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma', *Cancer*, 77(7), pp. 1303–1310. doi: 10.1002/(SICI)1097-0142(19960401)77:7<1303::AID-CNCR12>3.0.CO;2-5.
- Colombo, S. *et al.* (2011) 'Classical and Nonclassical Melanocytes in Vertebrates', in *Melanins and Melanosomes*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 21–61. doi: 10.1002/9783527636150.ch2.
- Condeelis, J. and Pollard, J. W. (2006) 'Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis', *Cell*, 124(2), pp. 263–266. doi: 10.1016/j.cell.2006.01.007.
- Corthay, A. (2014) 'Does the immune system naturally protect against cancer?', *Frontiers in Immunology*, 5(197), pp. 1–8. doi: 10.3389/fimmu.2014.00197.
- Costin, G.-E. and Hearing, V. J. (2007) 'Human skin pigmentation: melanocytes modulate skin color in response to stress', *The FASEB Journal*, 21(4), pp. 976–994. doi: 10.1096/fj.06-6649rev.
- Cruz-Muñoz, W. *et al.* (2012) 'Roles for endothelin receptor B and BCL2A1 in spontaneous CNS metastasis of melanoma', *Cancer Research*, 72(19), pp. 4909–4919. doi: 10.1158/0008-5472.CAN-12-2194.
- Curiel, T. J. *et al.* (2004) 'Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival', *Nature Medicine*, 10(9), pp. 942–949. doi: 10.1038/nm1093.
- Damsky, W. *et al.* (2015) 'mTORC1 activation blocks brafV600E-induced growth arrest but is insufficient for melanoma formation', *Cancer Cell*. Elsevier Inc., 27(1), pp. 41–56. doi: 10.1016/j.ccell.2014.11.014.
- Damsky, W. E. and Bosenberg, M. (2017) 'Melanocytic nevi and melanoma: unraveling a complex relationship', *Oncogene*. Nature Publishing Group, 36(42), pp. 5771–5792. doi: 10.1038/onc.2017.189.
- Damsky, W. E., Theodosakis, N. and Bosenberg, M. (2014) 'Melanoma metastasis: new concepts and evolving paradigms.', *Oncogene*. Nature Publishing Group, 33(19), pp. 2413–22. doi: 10.1038/onc.2013.194.
- Dankort, D. *et al.* (2009) 'Braf(V600E) cooperates with Pten loss to induce metastatic melanoma.', *Nature genetics*, 41(5), pp. 544–552. doi: 10.1038/ng.356.
- Davenport, A. P. *et al.* (2016) 'Endothelin', *Pharmacological Reviews*, 68(2), pp. 357–418. doi: 10.1124/pr.115.011833.
- Demunter, A. *et al.* (2001) 'Expression of the endothelin-B receptor in pigment cell lesions of the skin: Evidence for its role as tumor progression marker in malignant melanoma', *Virchows Archiv*, 438(5), pp. 485–491. doi: 10.1007/s004280000362.

- Dimanche-Boitrel, M. T. *et al.* (1994) 'In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining E-cadherin expression: An enhancing role of tumor-associated myofibroblasts', *International Journal of Cancer*, 56(4), pp. 512–521. doi: 10.1002/ijc.2910560410.
- Ding, L. *et al.* (1993) 'IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression', *Journal of Immunology*, 151(3), pp. 1224–1234.
- Dummer, W. *et al.* (1995) 'Elevated serum levels of interleukin-10 in patients with metastatic malignant melanoma', *Melanoma Res*, 5(1), pp. 67–68.
- Dunn, G. P., Old, L. J. and Schreiber, R. D. (2004) 'The Three Es of Cancer Immunoediting', *Annual Review of Immunology*, 22(1), pp. 329–360. doi: 10.1146/annurev.immunol.22.012703.104803.
- Eberle, J. *et al.* (1999) 'Downregulation of endothelin B receptor in human melanoma cell lines parallel to differentiation genes', *Journal of Investigative Dermatology*, 112(6), pp. 925–932. doi: 10.1046/j.1523-1747.1999.00598.x.
- Egeblad, M., Nakasone, E. S. and Werb, Z. (2010) 'Tumors as Organs: Complex Tissues that Interface with the Entire Organism', *Developmental Cell*. Elsevier Inc., 18(6), pp. 884–901. doi: 10.1016/j.devcel.2010.05.012.
- Ehrenreich, H. *et al.* (1990) 'Endothelins, peptides with potent vasoactive properties, are produced by human macrophages.', *The Journal of experimental medicine*, 172(6), pp. 1741–8. doi: 10.1084/jem.172.6.1741.
- Ejeta, G. *et al.* (2015) 'Genomic correlates of response to CTLA-4 blockade in metastatic melanoma', *Science*, 350(6257), pp. 207–212.
- Elder, D. *et al.* (1980) 'Dysplastic nevus syndrome: a phenotypic association of sporadic cutaneous melanoma', *Cancer*, 46(8), pp. 1787–94.
- Engle, S. J. *et al.* (2002) 'Elimination of colon cancer in germ-free transforming growth factor beta 1-deficient mice', *Cancer Research*, 62(22), pp. 6362–6366. doi: 10.1158/0008-5472.can-03-3255.
- Fang, H. and DeClerck, Y. A. (2013) 'Targeting the Tumor Microenvironment: From Understanding Pathways to Effective Clinical Trials', *Cancer Research*, 73(16), pp. 4965–4977. doi: 10.1158/0008-5472.CAN-13-0661.
- Fernandez, L. P. *et al.* (2009) 'Pigmentation-related genes and their implication in malignant melanoma susceptibility.', *Experimental dermatology*, 18(7), pp. 634–642. doi: 10.1111/j.1600-0625.2009.00846.x.
- Ferrone, S. and Marincola, F. M. (1995) 'Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance', *Immunology Today*, 16(10), pp. 487–494. doi: 10.1016/0167-5699(95)80033-6.

- Filep, J. G. *et al.* (1992) 'Endothelin-1 enhances vascular permeability in the rat heart through the ETA receptor', *European Journal of Pharmacology*, 219(2), pp. 343–344. doi: 10.1016/0014-2999(92)90318-X.
- Flier, J. S., Underhill, L. H. and Dvorak, H. F. (1986) 'Tumors: Wounds That Do Not Heal', *New England Journal of Medicine*, 315(26), pp. 1650–1659. doi: 10.1056/NEJM198612253152606.
- Frey, D. M. *et al.* (2010) 'High frequency of tumor-infiltrating FOXP3+regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients', *International Journal of Cancer*, 126(11), pp. 2635–2643. doi: 10.1002/ijc.24989.
- Fuertes, M. B. *et al.* (2008) 'Intracellular Retention of the NKG2D Ligand MHC Class I Chain-Related Gene A in Human Melanomas Confers Immune Privilege and Prevents NK Cell-Mediated Cytotoxicity', *The Journal of Immunology*, 180(7), pp. 4606–4614. doi: 10.4049/jimmunol.180.7.4606.
- Fukumura, D. *et al.* (1998) 'Tumor induction of VEGF promoter activity in stromal cells', *Cell*, 94(6), pp. 715–725. doi: 10.1016/S0092-8674(00)81731-6.
- Gabrilovich, D. I., Ostrand-Rosenberg, S. and Bronte, V. (2012) 'Coordinated regulation of myeloid cells by tumours', *Nature Reviews Immunology*. Nature Publishing Group, 12(4), pp. 253–268. doi: 10.1038/nri3175.
- Galon, J. *et al.* (2006) 'Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome', *Science*, 313(5795), pp. 1960–1964. doi: 10.1126/science.1129139.
- Garcia, R. J. *et al.* (2008) 'Endothelin 3 Induces Skin Pigmentation in a Keratin-Driven Inducible Mouse Model', *Journal of Investigative Dermatology*, 128(1), pp. 131–142. doi: 10.1038/sj.jid.5700948.
- Gassenmaier, M. *et al.* (2017) 'Serial or Parallel Metastasis of Cutaneous Melanoma? A Study of the German Central Malignant Melanoma Registry', *Journal of Investigative Dermatology*. Society for Investigative Dermatology, 137(12), pp. 2570–2577. doi: 10.1016/j.jid.2017.07.006.
- von Geldern, T. W. *et al.* (1999) 'Pyrrolidine-3-carboxylic acids as endothelin antagonists. 4. Side chain conformational restriction leads to ET(B) selectivity.', *Journal of medicinal chemistry*, 42(18), pp. 3668–78. doi: 10.1021/jm990170q.
- Giavina-Bianchi, M. H., Giavina-Bianchi Junior, P. F. and Festa Neto, C. (2017) 'Melanoma: tumor microenvironment and new treatments', *Anais Brasileiros de Dermatologia*, 92(2), pp. 156–166. doi: 10.1590/abd1806-4841.20176183.
- Giuliano, A. E., Moseley, H. S. and Morton, D. L. (1980) 'Clinical aspects of unknown primary melanoma', *Ann Surg*, 191(1), pp. 98–104. Available at: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1344625/pdf/annsurg00227-0112.pdf>.

- Gocheva, V. *et al.* (2010) 'IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion', *Genes and Development*, 24(3), pp. 241–255. doi: 10.1101/gad.1874010.
- Gong, D. *et al.* (2012) 'TGF β signaling plays a critical role in promoting alternative macrophage activation', *BMC Immunology*, 13(1), p. 31. doi: 10.1186/1471-2172-13-31.
- Gopalakrishnan, V. *et al.* (2018) 'Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients', *Science*, 359(6371), pp. 97–103. doi: 10.1126/science.aan4236.
- Gopalakrishnan, V. *et al.* (2018) 'The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy', *Cancer Cell*. Elsevier Inc., 33(4), pp. 570–580. doi: 10.1016/j.ccell.2018.03.015.
- Gorelik, L., Constant, S. and Flavell, R. A. (2002) 'Mechanism of Transforming Growth Factor β -induced Inhibition of T Helper Type 1 Differentiation', *The Journal of Experimental Medicine*, 195(11), pp. 1499–1505. doi: 10.1084/jem.20012076.
- Goswami, S. *et al.* (2005) 'Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop', *Cancer Research*, 65(12), pp. 5278–5283. doi: 10.1158/0008-5472.CAN-04-1853.
- Grimshaw, M. J., Wilson, J. L. and Balkwill, F. R. (2002) 'Endothelin-2 is a macrophage chemoattractant: Implications for macrophage distribution in tumors', *European Journal of Immunology*, 32(9), pp. 2393–2400. doi: 10.1002/1521-4141(200209)32:9<2393::AID-IMMU2393>3.0.CO;2-4.
- Grivennikov, S. I., Greten, F. R. and Karin, M. (2010) 'Immunity, Inflammation, and Cancer', *Cell*. Elsevier Inc., 140(6), pp. 883–899. doi: 10.1016/j.cell.2010.01.025.
- Groux, H. *et al.* (1998) 'Inhibitory and Stimulatory Effects of IL-10 on Human CD8+ T Cells', *The Journal of Immunology*, 160(7), pp. 3188–3193. Available at: <http://jimmunol.org/content/160/7/3188.abstract%5Cnhttp://www.jimmunol.org/content/160/7/3188.full.pdf%5Cnhttp://www.jimmunol.org/content/160/7/3188.long>.
- Guruli, G. *et al.* (2004) 'Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops', *Blood*, 104(7), pp. 2107–2115. doi: 10.1182/blood-2003-10-3559.
- Gutcher, I. and Becher, B. (2007) 'APC-derived cytokines and T cell polarization in autoimmune inflammation', *Journal of Clinical Investigation*, 117(5), pp. 1119–1127. doi: 10.1172/JCI31720.
- Haabeth, O. A. W., Bogen, B. and Corthay, A. (2012) 'A model for cancer-suppressive inflammation', *Onc Immunology*, 1(7), pp. 1146–1155. doi: 10.4161/onci.21542.

- Haanen, J. B. A. G. (2017) 'Converting Cold into Hot Tumors by Combining Immunotherapies', *Cell*. Elsevier Inc., 170(6), pp. 1055–1056. doi: 10.1016/j.cell.2017.08.031.
- Hagemann, T. *et al.* (2004) 'Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF- α dependent up-regulation of matrix metalloproteases', *Carcinogenesis*, 25(8), pp. 1543–1549. doi: 10.1093/carcin/bgh146.
- Hall, C. S. *et al.* (2012) 'Circulating Tumor Cells Melanoma Patients', *PLoS ONE*, 7(7), p. e41052. doi: 10.1016/j.jamcollsurg.2018.04.026.
- Hanada, T. *et al.* (2000) 'Prognostic value of tumor-associated macrophage count in human bladder cancer', *International Journal of Urology*, 7(7), pp. 263–269. doi: 10.1046/j.1442-2042.2000.00190.x.
- Hanahan, D. and Weinberg, R. A. (2011) 'Hallmarks of cancer: the next generation.', *Cell*. Elsevier Inc., 144(5), pp. 646–74. doi: 10.1016/j.cell.2011.02.013.
- Hanggono Achmad, T. and Rao, G. S. (1992) 'Chemotaxis of human blood monocytes toward endothelin-1 and the influence of calcium channel blockers', *Biochemical and Biophysical Research Communications*, 189(2), pp. 994–1000. doi: 10.1016/0006-291X(92)92302-E.
- Hawryluk, E. B. and Tsao, H. (2014) 'Melanoma : Clinical Features and Genomic Insights', *Cold Spring Harb Perspect Med*, 4(9), p. a015388. doi: 10.1101/cshperspect.a015388.
- Hayasaki, Y. *et al.* (1996) 'ICAM-1 expression on cardiac myocytes and aortic endothelial cells via their specific endothelin receptor subtype', *Biochemical and Biophysical Research Communications*, 229(3), pp. 817–824. doi: 10.1006/bbrc.1996.1886.
- Hida, K. *et al.* (2018) 'Contribution of tumor endothelial cells in cancer progression', *International Journal of Molecular Sciences*, 19(5), pp. 1–12. doi: 10.3390/ijms19051272.
- Hino, R. *et al.* (2010) 'Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma', *Cancer*, 116(7), pp. 1757–1766. doi: 10.1002/cncr.24899.
- Hodis, E. *et al.* (2012) 'A landscape of driver mutations in melanoma', *Cell*, 150(2), pp. 251–263. doi: 10.1016/j.cell.2012.06.024.
- Hosoda, K. *et al.* (1994) 'Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice', *Cell*, 79(7), pp. 1267–1276. doi: 10.1016/0092-8674(94)90017-5.

- Huang, B. *et al.* (2006) 'Gr-1+CD115+immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host', *Cancer Research*, 66(2), pp. 1123–1131. doi: 10.1158/0008-5472.CAN-05-1299.
- Huang, R. *et al.* (2012) 'Cytokine Antibody Arrays in Biomarker Discovery and Validation', *Current Proteomics*, 9(1), pp. 55–70. doi: 10.2174/157016412799746209.
- Ikeda, H., Old, L. J. and Schreiber, R. D. (2002) 'The roles of IFN γ in protection against tumor development and cancer immunoediting', *Cytokine and Growth Factor Reviews*, 13(2), pp. 95–109. doi: 10.1016/S1359-6101(01)00038-7.
- Imokawa, G. *et al.* (1997) 'The Role of Endothelin-1 in Epidermal Hyperpigmentation and Signaling Mechanisms of Mitogenesis and Melanogenesis', *Pigment Cell Res*, 10(4), pp. 218–28. doi: 10.1111/j.1600-0749.1997.tb00488.x.
- Imokawas, G., Yada, Y. and Miyagishi, M. (1992) 'Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes', *J Biol Chem*, 267(34), pp. 24675–24680.
- Ishikawa, K. *et al.* (1994) 'Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788.', *Proceedings of the National Academy of Sciences of the United States of America*, 91(May 1994), pp. 4892–4896. doi: 10.1073/pnas.91.11.4892.
- Iwata, S. *et al.* (2009) 'Regulation of endothelin-1-induced interleukin-6 production by Ca²⁺ influx in human airway smooth muscle cells', *European Journal of Pharmacology*. Elsevier B.V., 605(1–3), pp. 15–22. doi: 10.1016/j.ejphar.2008.12.045.
- Jamal, S. and Schneider, R. J. (2002) 'UV-induction of keratinocyte endothelin-1 downregulates E-cadherin in melanocytes and melanoma cells', *J Clin Invest*, 110(4), pp. 443–452. doi: 10.1172/JCI200213729.
- Jobin, C. (2018) 'Precision medicine using microbiota', *Science*, 359(6371), pp. 32–34. doi: 10.1126/science.aar2946.
- Kalluri, R. and Zeisberg, M. (2006) 'Fibroblasts in cancer', *Nature Reviews Cancer*, 6(5), pp. 392–401. doi: 10.1038/nrc1877.
- Kanitakis, J. (2002) 'Anatomy, histology and immunohistochemistry of normal human skin', *European Journal of Dermatology*, 12(4), pp. 390–401.
- Kawanabe, Y. and Nauli, S. M. (2011) 'Endothelin', *Cellular and Molecular Life Sciences*, 68(2), pp. 195–203. doi: 10.1007/s00018-010-0518-0.

- Kedzierski, R. M. and Yanagisawa, M. (2001) 'Endothelin System: The Double-Edged Sword in Health and Disease', *Annual Review of Pharmacology and Toxicology*, 41(1), pp. 851–876. doi: 10.1146/annurev.pharmtox.41.1.851.
- Kefford, R. *et al.* (2007) 'A Phase II study of bosentan, a dual endothelin receptor antagonist, as monotherapy in patients with stage IV metastatic melanoma', *Investigational New Drugs*, 25(3), pp. 247–252. doi: 10.1007/s10637-006-9014-7.
- Kefford, R. F. *et al.* (2010) 'A randomized, double-blind, placebo-controlled study of high-dose bosentan in patients with stage IV metastatic melanoma receiving first-line dacarbazine chemotherapy.', *Molecular cancer*, 9(69), pp. 1–9. doi: 10.1186/1476-4598-9-69.
- Khimji, A. karim and Rockey, D. C. (2010) 'Endothelin-Biology and disease', *Cellular Signalling*. Elsevier B.V., 22(11), pp. 1615–1625. doi: 10.1016/j.cellsig.2010.05.002.
- Kikuchi, K. *et al.* (1996) 'Decreased ET(B) receptor expression in human metastatic melanoma cells.', *Biochemical and biophysical research communications*, 219(3), pp. 734–739. doi: 10.1006/bbrc.1996.0303.
- Kim, R. *et al.* (2006) 'Tumor-Driven Evolution of Immunosuppressive Networks during Malignant Progression', *Cancer Research*, 66(11), pp. 5527–5536. doi: 10.1158/0008-5472.CAN-05-4128.
- Kingham, T. P. *et al.* (2010) 'Outcome of patients with a positive sentinel lymph node who do not undergo completion lymphadenectomy', *Annals of Surgical Oncology*, 17(2), pp. 514–520. doi: 10.1245/s10434-009-0836-3.
- Knol, A. C. *et al.* (2011) 'Prognostic value of tumor-infiltrating Foxp3+ T-cell subpopulations in metastatic melanoma', *Experimental Dermatology*, 20(5), pp. 430–434. doi: 10.1111/j.1600-0625.2011.01260.x.
- Kohan, D. E. (2006) 'The renal medullary endothelin system in control of sodium and water excretion and systemic blood pressure', *Current Opinion in Nephrology and Hypertension*, 15(1), pp. 34–40. doi: 10.1097/01.mnh.0000186852.15889.1a.
- Korn, E. L. *et al.* (2008) 'Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials', *Journal of Clinical Oncology*, 26(4), pp. 527–534. doi: 10.1200/JCO.2007.12.7837.
- Krauthammer, M. *et al.* (2015) 'Exome sequencing identifies recurrent mutations in NF1 and RASopathy genes in sun-exposed melanomas', *Nature Genetics*, 47(9), pp. 996–1002. doi: 10.1038/ng.3361.
- Kuilman, T. *et al.* (2008) 'Oncogene-Induced Senescence Relayed by an Interleukin-Dependent Inflammatory Network', *Cell*, 133(6), pp. 1019–1031. doi: 10.1016/j.cell.2008.03.039.

- Kumar, V. *et al.* (2016) 'The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment', *Trends in Immunology*. Elsevier Ltd, 37(3), pp. 208–220. doi: 10.1016/j.it.2016.01.004.
- Kumasaka, M. Y. *et al.* (2010) 'A Novel Mouse Model for De novo Melanoma', *Cancer Research*, 70(1), pp. 24–29. doi: 10.1158/0008-5472.CAN-09-2838.
- Kumasaka, M. Y. *et al.* (2015) 'Correlated expression levels of endothelin receptor B and Plexin C1 in melanoma', *American journal of cancer research*, 5(3), pp. 1117–1123.
- Lahav, R. *et al.* (1996) 'Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture.', *Proceedings of the National Academy of Sciences of the United States of America*, 93(9), pp. 3892–3897. doi: 10.1073/pnas.93.9.3892.
- Lahav, R. *et al.* (1998) 'Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors in vitro.', *Proceedings of the National Academy of Sciences of the United States of America*, 95(24), pp. 14214–14219. doi: 10.1073/pnas.95.24.14214.
- Lahav, R. *et al.* (2004) 'Endothelin receptor B inhibition triggers apoptosis and enhances angiogenesis in melanomas.', *Cancer research*, 64(24), pp. 8945–53. doi: 10.1158/0008-5472.CAN-04-1510.
- Lahav, R., Heffner, G. and Patterson, P. H. (1999) 'An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(20), pp. 11496–11500.
- Landskron, G. *et al.* (2014) 'Chronic Inflammation and Cytokines in the Tumor Microenvironment', *Journal of Immunology Research*, 2014, pp. 1–19. doi: 10.1155/2014/149185.
- LaPak, K. M. and Burd, C. E. (2014) 'The Molecular Balancing Act of p16INK4a in Cancer and Aging', *Molecular Cancer Research*, 12(2), pp. 167–183. doi: 10.1158/1541-7786.MCR-13-0350.
- Larkin, J. *et al.* (2015) 'Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma', *New England Journal of Medicine*, 373(1), pp. 23–34. doi: 10.1056/NEJMoa1504030.
- Lawrence, M. S. *et al.* (2013) 'Mutational heterogeneity in cancer and the search for new cancer-associated genes', *Nature*, 499(7457), pp. 214–218. doi: 10.1038/nature12213.
- Leach, D. R., Krummel, M. F. and Allison, J. P. (1996) 'Enhancement of Antitumor Immunity by CTLA-4 Blockade', *Science*, 271(5256), pp. 1734–1736. doi: 10.1126/science.271.5256.1734.

- Ledford, H. (2014) 'Cancer treatment: The killer within', *Nature*, 508(7494), pp. 24–26. doi: 10.1038/508024a.
- Lee, G. R. (2017) 'Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells', *Mediators of inflammation*, 2017, pp. 1–9. doi: 10.1155/2017/5458178.
- Lee, H., Levorse, J. M. and Shin, M. K. (2003) 'The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors', *Dev Biol.*, 259(1), pp. 162–175. doi: 10.1016/S0012-1606(03)00160-X.
- Liao, D. *et al.* (2009) 'Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model', *PLoS ONE*, 4(11), p. e7965. doi: 10.1371/journal.pone.0007965.
- Liu, C. *et al.* (2007) 'Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host', *Blood*, 109(10), pp. 4336–4342. doi: 10.1182/blood-2006-09-046201.
- Liu, C., Workman, C. J. and Vignali, D. A. A. (2016) 'Targeting regulatory T cells in tumors', *FEBS Journal*, 283, pp. 2731–2748. doi: 10.1111/febs.13656.
- Lo, J. A. and Fisher, D. E. (2014) 'The melanoma revolution: from UV carcinogenesis to a new era in therapeutics.', *Science*, 346(6212), pp. 945–9. doi: 10.1126/science.1253735.
- López Farré, A. *et al.* (1993) 'Effect of endothelin-1 on neutrophil adhesion to endothelial cells and perfused heart', *Circulation*, 88(3), pp. 1166–1171. doi: 10.1161/01.CIR.88.3.1166.
- Maggi, L. B. *et al.* (2014) 'ARF tumor suppression in the nucleolus', *Biochimica et Biophysica Acta - Molecular Basis of Disease*. Elsevier B.V., 1842(6), pp. 831–839. doi: 10.1016/j.bbadis.2014.01.016.
- Maishi, N. *et al.* (2016) 'Tumour endothelial cells in high metastatic tumours promote metastasis via epigenetic dysregulation of biglycan', *Scientific Reports*. Nature Publishing Group, 6(28039). doi: 10.1038/srep28039.
- Maishi, N. and Hida, K. (2017) 'Tumor endothelial cells accelerate tumor metastasis', *Cancer Science*, 108(10), pp. 1921–1926. doi: 10.1111/cas.13336.
- Mannino, M. H. *et al.* (2015) 'The paradoxical role of IL-10 in immunity and cancer', *Cancer Letters*. Elsevier Ireland Ltd, 367(2), pp. 103–107. doi: 10.1016/j.canlet.2015.07.009.
- Mantovani, A. *et al.* (2008) 'Tumour immunity: effector response to tumour and role of the microenvironment', *The Lancet*, 371(9614), pp. 771–783. doi: 10.1016/S0140-6736(08)60241-X.

- Marcoe, J. P. *et al.* (2012) 'TGF- β is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy', *Nature Immunology*, 13(9), pp. 843–850. doi: 10.1038/ni.2388.
- Masaki, T. (2004) 'Historical review: Endothelin', *Trends in Pharmacological Sciences*, 25(4), pp. 219–224. doi: 10.1016/j.tips.2004.02.008.
- Massagué, J. (2008) 'TGF β in Cancer', *Cell*, 134(2), pp. 215–230. doi: 10.1016/j.cell.2008.07.001.
- Matson, V. *et al.* (2018) 'The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients', *Science*, 359(6371), pp. 104–108. doi: 10.1126/science.aao3290.
- Melnikova, V. O. and Bar-Eli, M. (2009) 'Inflammation and melanoma metastasis', *Pigment Cell & Melanoma Research*, 22(3), pp. 257–267. doi: 10.1111/j.1755-148X.2009.00570.x.
- Moretti, S. *et al.* (1999) 'Immunohistochemical evidence of cytokine networks during progression of human melanocytic lesions', *International Journal of Cancer*, 84(2), pp. 160–168. doi: 10.1002/(SICI)1097-0215(19990420)84:2<160::AID-IJC12>3.0.CO;2-R.
- Morvan, M. G. and Lanier, L. L. (2016) 'NK cells and cancer: You can teach innate cells new tricks', *Nature Reviews Cancer*. Nature Publishing Group, 16(1), pp. 7–19. doi: 10.1038/nrc.2015.5.
- Moss, R. B. *et al.* (2004) 'Th1/Th2 cells in inflammatory disease states: therapeutic implications', *Expert Opinion on Biological Therapy*, 4(12), pp. 1887–1896. doi: 10.1517/14712598.4.12.1887.
- Muenst, S. *et al.* (2016) 'The immune system and cancer evasion strategies: Therapeutic concepts', *Journal of Internal Medicine*, 279(6), pp. 541–562. doi: 10.1111/joim.12470.
- Mulé, J. J. *et al.* (1992) 'Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice', *J Immunol*, 148(8), pp. 2622–2629.
- Murakami, T., Cardones, A. R. and Hwang, S. T. (2004) 'Chemokine receptors and melanoma metastasis.', *Journal of dermatological science*, 36(2), pp. 71–8. doi: 10.1016/j.jdermsci.2004.03.002.
- Nagarsheth, N., Wicha, M. S. and Zou, W. (2017) 'Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy', *Nature Reviews Immunology*. Nature Publishing Group, 17(9), pp. 559–572. doi: 10.1038/nri.2017.49.
- Nemunaitis, J. *et al.* (2001) 'Comparison of Serum Interleukin-10 (IL-10) Levels Between Normal Volunteers and Patients with Advanced Melanoma', *Cancer Investigation*, 19(3), pp. 239–247. doi: 10.1081/CNV-100102550.

- Nett, P. C. *et al.* (2006) 'Recent developments on endothelin antagonists as immunomodulatory drugs--from infection to transplantation medicine.', *Recent patents on cardiovascular drug discovery*, 1(3), pp. 265–276. doi: 10.2174/157489006778776990.
- Noonan, F. P. *et al.* (2012) 'Melanoma induction by ultraviolet A but not ultraviolet B radiation requires melanin pigment', *Nature Communications*. Nature Publishing Group, 3(May), pp. 810–884. doi: 10.1038/ncomms1893.
- Novitskiy, S. V. *et al.* (2012) 'Deletion of TGF- signaling in myeloid cells enhances their anti-tumorigenic properties', *Journal of Leukocyte Biology*, 92(3), pp. 641–651. doi: 10.1189/jlb.1211639.
- Oft, M. (2014) 'IL-10: Master Switch from Tumor-Promoting Inflammation to Antitumor Immunity', *Cancer Immunology Research*, 2(3), pp. 194–199. doi: 10.1158/2326-6066.CIR-13-0214.
- Ohga, N. *et al.* (2012) 'Heterogeneity of tumor endothelial cells: Comparison between tumor endothelial cells isolated from high- and low-metastatic tumors', *American Journal of Pathology*. Elsevier Inc., 180(3), pp. 1294–1307. doi: 10.1016/j.ajpath.2011.11.035.
- Olumi, A. F. *et al.* (1999) 'Carcinoma-associated Fibroblasts Direct Tumor Progression of Initiated Human Prostatic Epithelium', *Cancer Res*, 59(19), pp. 5002–5011.
- Omholt, K. *et al.* (2002) 'Screening of N-ras codon 61 mutations in paired primary and metastatic cutaneous melanomas: Mutations occur early and persist throughout tumor progression', *Clinical Cancer Research*, 8(11), pp. 3468–3474. doi: 10.1002/sce.20023.
- Omholt, K. *et al.* (2003) 'NRAS and BRAF Mutations Arise Early during Melanoma Pathogenesis and Are Preserved throughout Tumor Progression', *Clinical Cancer Research*, 9(17), pp. 6483–6488. doi: 10.1364/OFT.2014.OW1B.3.
- Ozaki, K. and Leonard, W. J. (2002) 'Cytokine and cytokine receptor pleiotropy and redundancy', *Journal of Biological Chemistry*, 277(33), pp. 29355–29358. doi: 10.1074/jbc.R200003200.
- Palucka, A. K. and Coussens, L. M. (2016) 'The Basis of Oncoimmunology', *Cell*. Elsevier Inc., 164(6), pp. 1233–1247. doi: 10.1016/j.cell.2016.01.049.
- Paluncic, J. *et al.* (2016) 'Roads to melanoma: Key pathways and emerging players in melanoma progression and oncogenic signaling', *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier B.V., 1863(4), pp. 770–784. doi: 10.1016/j.bbamcr.2016.01.025.
- Parato, K. G. *et al.* (2002) 'Normalization of natural killer cell function and phenotype with effective anti-HIV therapy and the role of IL-10', *Aids*, 16(9), pp. 1251–1256. doi: 10.1097/00002030-200206140-00007.

- Passarelli, A. *et al.* (2017) 'Immune system and melanoma biology: a balance between immunosurveillance and immune escape', *Oncotarget*, 8(62), pp. 106132–106142. doi: 10.18632/oncotarget.22190.
- Pavan, W. J. and Tilghman, S. M. (1994) 'Piebald lethal (sl) acts early to disrupt the development of neural crest-derived melanocytes', *Proc Natl Acad Sci U S A.*, 91(15), pp. 7159–7163. doi: 10.1073/pnas.91.15.7159.
- Peppas, D. *et al.* (2010) 'Blockade of Immunosuppressive Cytokines Restores NK Cell Antiviral Function in Chronic Hepatitis B Virus Infection', *PLoS Pathogens*. Edited by L. G. Guidotti, 6(12), p. e1001227. doi: 10.1371/journal.ppat.1001227.
- Petersen, O. W. *et al.* (2003) 'Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma', *American Journal of Pathology*, 162(2), pp. 391–402. doi: 10.1016/S0002-9440(10)63834-5.
- Pickup, M., Novitskiy, S. and Moses, H. L. (2013) 'The roles of TGF β in the tumour microenvironment', *Nature Reviews Cancer*. Nature Publishing Group, 13(11), pp. 788–799. doi: 10.1038/nrc3603.
- Pollock, P. M. *et al.* (2003) 'High frequency of BRAF mutations in nevi', *Nature Genetics*, 33(1), pp. 19–20. doi: 10.1038/ng1054.
- Postow, M. A., Callahan, M. K. and Wolchok, J. D. (2015) 'Immune checkpoint blockade in cancer therapy', *Journal of Clinical Oncology*, 33(17), pp. 1974–1982. doi: 10.1200/JCO.2014.59.4358.
- Prall, F. *et al.* (2004) 'Prognostic role of CD8+ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability', *Human Pathology*, 35(7), pp. 808–816. doi: 10.1016/j.humpath.2004.01.022.
- Qian, B. Z. *et al.* (2011) 'CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis', *Nature*. Nature Publishing Group, 475(7355), pp. 222–225. doi: 10.1038/nature10138.
- Quail, D. F. and Joyce, J. A. (2013) 'Microenvironmental regulation of tumor progression and metastasis', *Nature Medicine*, 19(11), pp. 1423–1437. doi: 10.1038/nm.3394.
- Reid, A. L. *et al.* (2013) 'Markers of circulating tumour cells in the peripheral blood of patients with melanoma correlate with disease recurrence and progression', *British Journal of Dermatology*, 168(1), pp. 85–92. doi: 10.1111/bjd.12057.
- Reid, K. *et al.* (1996) 'Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation.', *Development*, 122(12), pp. 3911–3919.

- Reifenberger, J. *et al.* (2000) 'Allelic losses on chromosome arm 10q and mutation of the PTEN (MMAC1) tumour suppressor gene in primary and metastatic malignant melanomas', *Virchows Archiv*, 436(5), pp. 487–493. doi: 10.1007/s004280050477.
- Rhee, I. (2016) 'Diverse macrophages polarization in tumor microenvironment', *Archives of Pharmacal Research*. Pharmaceutical Society of Korea, 39(11), pp. 1588–1596. doi: 10.1007/s12272-016-0820-y.
- Ribas, A. *et al.* (2017) 'Oncolytic Virotherapy Promotes Intratumoral T Cell Infiltration and Improves Anti-PD-1 Immunotherapy', *Cell*. Elsevier, 170(6), p. 1109–1119.e10. doi: 10.1016/j.cell.2017.08.027.
- Ribas, A. and Wolchok, J. D. (2018) 'Cancer immunotherapy using checkpoint blockade', *Science*, 359(6382), pp. 1350–1355. doi: 10.1126/science.aar4060.
- Richmond, A., Yang, J. and Su, Y. (2009) 'The good and the bad of chemokines/chemokine receptors in melanoma.', *Pigment cell & melanoma research*, 22(2), pp. 175–86. doi: 10.1111/j.1755-148X.2009.00554.x.
- Robert, C. *et al.* (2011) 'Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma', *New England Journal of Medicine*, 364(26), pp. 2517–2526. doi: 10.1056/NEJMoa1104621.
- Roder, J. C. *et al.* (1980) 'A new immunodeficiency disorder in humans involving NK cells', *Nature*, 284(5756), pp. 553–555. doi: 10.1038/284553a0.
- Rosanò, L. *et al.* (2004) 'Endothelin-B receptor blockade inhibits molecular effectors of melanoma cell progression.', *Journal of cardiovascular pharmacology*, 44(November), pp. S136–S139. doi: 10.1097/01.fjc.0000166247.35992.dd.
- Rosanò, L., Spinella, F. and Bagnato, A. (2013) 'Endothelin 1 in cancer: biological implications and therapeutic opportunities.', *Nature reviews. Cancer*. Nature Publishing Group, 13(9), pp. 1–15. doi: 10.1038/nrc3546.
- Saenger, Y. M. and Wolchok, J. D. (2008) 'The heterogeneity of the kinetics of response to ipilimumab in metastatic melanoma: Patient cases', *Cancer Immunity*, 8(October 2007), pp. 1–7. doi: 080102 [pii].
- Saldana-Caboverde, A. and Kos, L. (2010) 'Roles of endothelin signaling in melanocyte development and melanoma', *Pigment Cell and Melanoma Research*, 23(2), pp. 160–170. doi: 10.1111/j.1755-148X.2010.00678.x.
- Sanborn, J. Z. *et al.* (2015) 'Phylogenetic analyses of melanoma reveal complex patterns of metastatic dissemination', *Proceedings of the National Academy of Sciences*, 112(35), pp. 10995–11000. doi: 10.1073/pnas.1508074112.
- Sato, T. *et al.* (2011) 'Interleukin 10 in the tumor microenvironment: A target for anticancer immunotherapy', *Immunologic Research*, 51(2–3), pp. 170–182. doi: 10.1007/s12026-011-8262-6.

Schadendorf, D., Fisher, D. E., *et al.* (2015) 'Melanoma', *Nature Reviews Disease Primers*, 1(15003). doi: 10.1038/nrdp.2015.3.

Schadendorf, D., Hodi, F. S., *et al.* (2015) 'Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma', *Journal of Clinical Oncology*, 33(17), pp. 1889–1894. doi: 10.1200/JCO.2014.56.2736.

Schauer, E. *et al.* (1994) 'Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes', *Journal of Clinical Investigation*, 93(5), pp. 2258–2262. doi: 10.1172/JCI117224.

Schreiber, R. D., Old, L. J. and Smyth, M. J. (2011) 'Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion', *Science*, 331(6024), pp. 1565–1570. doi: 10.1126/science.1203486.

Scott, G. a *et al.* (2009) 'Plexin C1, a receptor for semaphorin 7a, inactivates cofilin and is a potential tumor suppressor for melanoma progression.', *The Journal of investigative dermatology*. Elsevier Masson SAS, 129(4), pp. 954–963. doi: 10.1038/jid.2008.329.

Seder, R. A. *et al.* (1993) 'Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming.', *Proceedings of the National Academy of Sciences*, 90(21), pp. 10188–10192. doi: 10.1073/pnas.90.21.10188.

Sekulic, A. *et al.* (2010) 'Activating BRAF mutations in eruptive melanocytic naevi', *British Journal of Dermatology*, 163(5), pp. 1095–1098. doi: 10.1111/j.1365-2133.2010.09989.x.

Shain, A. H. *et al.* (2015) 'The Genetic Evolution of Melanoma from Precursor Lesions', *New England Journal of Medicine*, 373(20), pp. 1926–1936. doi: 10.1056/NEJMoa1502583.

Shain, A. H. and Bastian, B. C. (2016) 'From melanocytes to melanomas', *Nat Rev Cancer*. Nature Publishing Group, 16(6), pp. 345–358. doi: 10.1038/nrc.2016.37.

Shankaran, V. *et al.* (2001) 'IFN γ and lymphocytes prevent primary tumor development and shape tumour immunogenicity', *Nature*, 410(6832), pp. 1107–1111. doi: 10.1038/35074122.

Sherwood, L. M., Parris, E. E. and Folkman, J. (1971) 'Tumor Angiogenesis: Therapeutic Implications', *New England Journal of Medicine*, 285(21), pp. 1182–1186. doi: 10.1056/NEJM197111182852108.

Shinagawa, S. *et al.* (2017) 'T cells upon activation promote endothelin 1 production in monocytes via IFN- γ and TNF- α ', *Scientific Reports*. Springer US, 7(1), pp. 1–8. doi: 10.1038/s41598-017-14202-5.

- Shitara, D. *et al.* (2014) 'Nevus-associated melanomas: Clinicopathologic features', *American Journal of Clinical Pathology*, 142(4), pp. 485–491. doi: 10.1309/AJCP4L5CJGKTJVDD.
- Sica, A., Allavena, P. and Mantovani, A. (2008) 'Cancer related inflammation: The macrophage connection', *Cancer Letters*, 267(2), pp. 204–215. doi: 10.1016/j.canlet.2008.03.028.
- Simonson, M. S. and Dunn, M. J. (1990) 'Cellular signaling by peptides of the endothelin gene family.', *FASEB J.*, 4(12), pp. 2989–3000. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2168326>.
- Sinha, P., Clements, V. K. and Ostrand-Rosenberg, S. (2005) 'Reduction of Myeloid-Derived Suppressor Cells and Induction of M1 Macrophages Facilitate the Rejection of Established Metastatic Disease', *The Journal of Immunology*, 174(2), pp. 636–645. doi: 10.4049/jimmunol.174.2.636.
- Sivan, A. *et al.* (2015) 'Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy', *Science*, 350(6264), pp. 1084–1089. doi: 10.1126/science.aac4255.
- Smith, H. A. and Kang, Y. (2013) 'The Metastasis-Promoting Roles of Tumor-Associated Immune Cells', *Journal of Molecular Medicine*, 91(4), pp. 411–429. doi: 10.1007/s00109-013-1021-5.
- Smith, M. P. *et al.* (2017) 'Targeting endothelin receptor signalling overcomes heterogeneity driven therapy failure', *EMBO Molecular Medicine*, 9(8), pp. 1011–1029. doi: 10.15252/emmm.
- Smyth, M. J. *et al.* (1998) 'An essential role for tumor necrosis factor in natural killer cell-mediated tumor rejection in the peritoneum', *Journal of Experimental Medicine*, 188(9), pp. 1611–1619. doi: 10.1016/j.carbon.2013.09.012.
- De Snoo, F. A. and Hayward, N. K. (2005) 'Cutaneous melanoma susceptibility and progression genes', *Cancer Letters*, 230(2), pp. 153–186. doi: 10.1016/j.canlet.2004.12.033.
- Soufir, N. *et al.* (2005) 'Association between endothelin receptor B nonsynonymous variants and melanoma risk', *Journal of the National Cancer Institute*, 97(17), pp. 1297–1301. doi: 10.1093/jnci/dji253.
- Spano, D. and Zollo, M. (2012) 'Tumor microenvironment: a main actor in the metastasis process.', *Clinical & experimental metastasis*, 29(4), pp. 381–95. doi: 10.1007/s10585-012-9457-5.
- Spica, T. *et al.* (2011) 'EDNRB gene variants and melanoma risk in two southern European populations', *Clinical and experimental dermatology*, 36(7), pp. 782–787. doi: 10.1111/j.1365-2230.2011.04062.x.

- Spinella, F. *et al.* (2007) 'Endothelin-1 and endothelin-3 promote invasive behavior via hypoxia-inducible factor-1 α in human melanoma cells', *Cancer Research*, 67(4), pp. 1725–1734. doi: 10.1158/0008-5472.CAN-06-2606.
- Spinella, F. *et al.* (2010) 'Endothelin-1 inhibits prolyl hydroxylase domain 2 to activate hypoxia-inducible factor-1 α in melanoma cells.', *PloS one*, 5(6), p. e11241. doi: 10.1371/journal.pone.0011241.
- Spranger, S., Bao, R. and Gajewski, T. F. (2015) 'Melanoma-intrinsic β -catenin signalling prevents anti-tumour immunity', *Nature*, 523(7559), pp. 231–235. doi: 10.1038/nature14404.
- Stewart, T. *et al.* (1995) 'Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation', *The Lancet*, 346(8978), pp. 796–798. doi: 10.1016/S0140-6736(95)91618-0.
- Stretch, J. R. *et al.* (1991) 'Expression of Mutant p53 in Melanoma', *Cancer Research*, 51(21), pp. 5976–5979.
- Sumimoto, H. *et al.* (2006) 'The BRAF–MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells', *The Journal of Experimental Medicine*, 203(7), pp. 1651–1656. doi: 10.1084/jem.20051848.
- Swain, S. L. *et al.* (1990) 'IL-4 directs the development of Th2-like helper effectors', *The Journal of Immunology*, 145(11), pp. 3796–3800.
- Taga, K. and Tosato, G. (1992) 'IL-10 inhibits cytokine production by activated macrophages', *J Immunol*, 148(4), pp. 1143–8.
- Talmadge, J. E. *et al.* (1980) 'Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice', *Journal of the National Cancer Institute*, 65(5), pp. 929–935.
- Tang, L. *et al.* (2008) 'Endothelin-3 is produced by metastatic melanoma cells and promotes melanoma cell survival', *Journal of Cutaneous Medicine and Surgery*, 12(2), pp. 64–70. doi: 10.2310/7750.2008.06164.
- Taube, J. M. *et al.* (2009) 'Benign nodal nevi frequently harbor the activating V600E BRAF mutation', *American Journal of Surgical Pathology*, 33(4), pp. 568–571. doi: 10.1097/PAS.0b013e31818a64fb.
- TCGA (2015) 'Genomic Classification of Cutaneous Melanoma', *Cell*, 161(7), pp. 1681–1696. doi: 10.1016/j.cell.2015.05.044.
- Thirumaran, R. K. *et al.* (2006) 'Re: Association Between Endothelin Receptor B Nonsynonymous Variants and Melanoma Risk', *JNCI Journal of the National Cancer Institute*, 98(17), pp. 1252–1253. doi: 10.1093/jnci/djj336.
- Thomas, D. A. and Massagué, J. (2005) 'TGF- β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance', *Cancer Cell*, 8(5), pp. 369–380. doi: 10.1016/j.ccr.2005.10.012.

- Tirosh, I. *et al.* (2016) 'Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq.', *Science*, 352(6282), pp. 189–96. doi: 10.1126/science.aad0501.
- Topalian, S. L. *et al.* (2014) 'Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab', *Journal of Clinical Oncology*, 32(10), pp. 1020–1030. doi: 10.1200/JCO.2013.53.0105.
- Tsao, H. *et al.* (2003) 'The transformation rate of moles (melanocytic nevi) into cutaneous melanoma: A population-based estimate', *Archives of Dermatology*, 139(3), pp. 282–288. doi: 10.1001/archderm.139.3.282.
- Tsao, H. *et al.* (2004) 'Genetic Interaction between NRAS and BRAF Mutations and PTEN/MMAC1 Inactivation in Melanoma', *Journal of Investigative Dermatology*. Elsevier Masson SAS, 122(2), pp. 337–341. doi: 10.1046/j.0022-202X.2004.22243.x.
- Uchida, Y. *et al.* (1988) 'Endothelin, a novel vasoconstrictor peptide, as potent bronchoconstrictor', *European Journal of Pharmacology*, 154(2), pp. 227–228. doi: 10.1016/S0140-6736(56)90268-9.
- Umansky, V. and Sevko, A. (2012) 'Melanoma-induced immunosuppression and its neutralization', *Seminars in Cancer Biology*. Elsevier Ltd, 22(4), pp. 319–326. doi: 10.1016/j.semcancer.2012.02.003.
- Veldhoen, M. *et al.* (2006) 'TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells', *Immunity*, 24(2), pp. 179–189. doi: 10.1016/j.immuni.2006.01.001.
- Vetizou, M. *et al.* (2015) 'Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota', *Science*, 350(6264), pp. 1079–1084. doi: 10.1126/science.aad1329.
- Villanueva, J. and Herlyn, M. (2008) 'Melanoma and the tumor microenvironment.', *Current oncology reports*, 10(5), pp. 439–446. doi: 10.1007/s11912-008-0067-y.
- de Waal Malefyt, R. *et al.* (1991) 'Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression', *Journal of Experimental Medicine*, 174(4), pp. 915–924. doi: 10.1084/jem.174.4.915.
- Wang, J. *et al.* (2017) 'UV-induced somatic mutations elicit a functional T cell response in the YUMMER1.7 Mouse Melanoma Model', *Pigment Cell & Melanoma Research*, 30(4), pp. 428–435. doi: 10.1111/pcmr.12591.
- Wilson, S. H., Simari, R. D. and Lerman, A. (2001) 'The effect of endothelin-1 on nuclear factor kappa B in macrophages', *Biochemical and Biophysical Research Communications*, 286(5), pp. 968–972. doi: 10.1006/bbrc.2001.5485.

- Wouters, J. *et al.* (2015) 'First-in-Human Proof-of-Concept Study: Intralesional Administration of BQ788, an Endothelin Receptor B Antagonist, to Melanoma Skin Metastases', *The Oncologist*, 20(10), pp. 1121–1122. doi: 10.1634/theoncologist.2015-0139.
- Wu, M. H. *et al.* (2012) 'Endothelin-1 promotes MMP-13 production and migration in human chondrosarcoma cells through FAK/PI3K/Akt/mTOR pathways', *Journal of Cellular Physiology*, 227(8), pp. 3016–3026. doi: 10.1002/jcp.23043.
- Yanagisawa, M. *et al.* (1988) 'A novel potent vasoconstrictor peptide produced by vascular endothelial cells', *Nature*, 332(6163), pp. 411–415. doi: 10.1038/332411a0.
- Yang, L. L. *et al.* (2004) 'Conditional Cardiac Overexpression of Endothelin-1 Induces Inflammation and Dilated Cardiomyopathy in Mice', *Circulation*, 109(2), pp. 255–261. doi: 10.1161/01.CIR.0000105701.98663.D4.
- Yang, W. *et al.* (2008) 'PD-L1: PD-1 interaction contributes to the functional suppression of T-cell responses to human uveal melanoma cells in vitro', *Investigative Ophthalmology and Visual Science*, 49(6), pp. 2518–2525. doi: 10.1167/iovs.07-1606.
- Zarour, H. M. (2016) 'Reversing T-cell dysfunction and exhaustion in cancer', *Clinical Cancer Research*, 22(8), pp. 1856–1864. doi: 10.1158/1078-0432.CCR-15-1849.
- Zeisberg, E. M. *et al.* (2007) 'Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts', *Cancer Research*, 67(21), pp. 10123–10128. doi: 10.1158/0008-5472.CAN-07-3127.

CHAPTER

2. Expression of Ednrb in Melanoma Microenvironment

2.1. Introduction

Expression of endothelins and their cognate receptors have been detected in a variety of tumor types (Nelson *et al.*, 2003). Colon (Egidy, Juillerat-Jeanneret, *et al.*, 2000), lung (Giaid *et al.*, 1990), bladder (Said *et al.*, 2011), prostate (Gohji *et al.*, 2001) ovarian (Rosanò, Spinella and Bagnato, 2010) and breast (Grimshaw, 2005) cancers are examples of carcinomas that express components of the endothelin signaling pathway. Glioblastoma (Egidy, Eberl, *et al.*, 2000) and melanoma (Demunter *et al.*, 2001) are non-carcinomas that also express endothelin. As previously discussed in Chapter 1, it has been clearly demonstrated that tumor cells express endothelin. It is has also become evident that endothelin is not only expressed by tumor cells but also by multiple populations of stromal cells in the TME including fibroblasts, endothelial and immune cells (Rosanò, Spinella and Bagnato, 2013; Rosanò and Bagnato, 2016). It is also widely accepted that stromal cells in the TME are major players during tumor development. Based on these findings endothelin has been proposed as an important modulator of tumor-stromal cells interactions in the TME (Binder *et al.*, 2009).

Very interestingly, the expression pattern of endothelins and their cognate receptors in the TME is different when epithelial and non-epithelial tumors are compared. In carcinomas, the main source of endothelin ligands in the TME is usually the tumor cells themselves. With respect to the receptors, there is some variability in that Ednra is mostly expressed by cancer cells such as in prostate, ovarian and breast cancer whereas the expression of Ednra is generally detected

in other cells inside the TME such as in glioblastoma (Aubert and Juillerat-Jeanneret, 2016).

The non-carcinoma type of cancer for which the endothelin pathway has been best characterized is glioblastoma. In glioblastoma, stromal cells express *Ednra* and are the main source of endothelin while tumors cells express *Ednrb* (Egidy, Eberl, *et al.*, 2000). Although melanoma is considered a non-carcinoma type of tumor, it may also share some features with carcinomas because melanocytes originate from ectodermal-derived neural crest cells (Braeuer *et al.*, 2014). Melanoma cells express EDNRB (Yohn *et al.*, 1994; Lahav, Heffner and Patterson, 1999; Bagnato *et al.*, 2004; Asundi *et al.*, 2011) as well as secrete EDN1 (Chiriboga *et al.*, 2016) and EDN3 (Tang *et al.*, 2008). It is not clear whether stromal cells respond to the endothelin available in the melanoma microenvironment. Recently, Chiriboga and colleagues detected the expression of EDN1 in macrophages found in melanoma tumors. Yet, the authors did not address the expression of endothelin receptors in stromal cells (Chiriboga *et al.*, 2016).

Regardless of the embryonic origin of cancer cells, endothelin signaling plays diverse roles in the TME (Figure 2.1). In the TME, the endothelin pathway has been mostly evaluated in fibroblasts and endothelial cells. Endothelin participates in a bidirectional stimulatory loop involving breast cancer cells and fibroblasts in the TME. Tumor cells secrete EDN1 that stimulates the production of inflammatory mediator PGE₂ in fibroblasts. In presence of pro-inflammatory cytokine IL-1, PGE₂ induces EDN1 production in tumor cells (Patel, Sheth and Schrey, 1997).

Endothelin signaling mediates the conversion of quiescent fibroblasts into cancer-associated fibroblasts (CAFs) in colon cancer, which is crucial for the creation of the permissive stroma for tumor cells. *In vitro* activation of each endothelin receptor produces different outcomes in CAFs and blockage of endothelin receptors prevents CAFs activity. Activation of EDNRA promotes cell growth while EDNRB stimulation is responsible for CAFs migration. Both EDNRA and EDNRB regulates CAFs contraction. Additionally, EDN1 induces the expression of profibrotic genes, secretion of ECM components and MMP-2 (Knowles *et al.*, 2012).

The connection between endothelin and endothelial cells in the TME has been addressed in many studies. Endothelin directly and indirectly promotes angiogenesis in the TME. EDNRB activation by EDN1 promotes endothelial cell proliferation, migration, and invasion (Salani, Taraboletti, *et al.*, 2000). Binding of EDN1 on EDNRA expressed by tumor cells results in the secretion of VEGF, the master regulator of angiogenesis (Salani, Castro, *et al.*, 2000). Activation of endothelin signaling in blood vessels functions as an important regulator of immune cell recruitment to the TME. In fact, EDNRA and EDNRB show contrasting effects in the recruitment of tumor-specific lymphocytes. Overexpression of EDNRB in endothelial cells from aggressive glioblastomas and ovarian cancer is associated with less cytotoxic T lymphocytes (CTLs) infiltration (Buckanovich *et al.*, 2008) and more regulatory T cells (Tregs) present in the TME. These data suggest that high expression of EDNRB in TME cells impairs recruitment of tumor inhibitory immune cells while contributing to evasion of immune detection (Nakashima *et al.*, 2016). EDNRB blockage increases the expression of adhesion

molecules ICAM-1 in the vasculature, which in turn enhances lymphocytes homing to the TME (Buckanovich *et al.*, 2008). Ovarian tumors treated with dual EDNRA/EDNRB inhibitor showed an unexpected decrease in ICAM-1 expression and consequently a reduced amount of CD8⁺ infiltrate. This finding implicates that EDNRA activation is essential for anti-tumor cells recruitment while stimulation of EDNRB prevents CTL mediated responses against tumors (Coffman *et al.*, 2013). Accordingly, EDNRB blockage alone might be more beneficial to patients than simultaneous inhibition of EDNRA and EDNRB.

Endothelin is considered a chemoattractant for macrophages to the TME. EDN1 produced by bladder cancer cells promotes macrophage recruitment via chemokine CCL2 production (Said *et al.*, 2011). Beyond chemotaxis, endothelin also increases the expression of genes associated with macrophage activation (Grimshaw, Wilson and Balkwill, 2002). Tumor-derived EDN1 stimulates EDNRA activation in tumor-associated macrophages (TAMs) resulting in production of pro-inflammatory cytokines TNF- α , IL-6 and COX-2 (Said *et al.*, 2011). TAMs also induce activation of endothelin signaling in endothelial and breast cancer cells. This activation promotes the expression of adhesion molecules resulting in cancer-endothelial cell adhesion and transendothelial migration (Chen *et al.*, 2014). Dendritic cell (DC) is another type of antigen presenting cell (APC) of which function is regulated by endothelin signaling. Remarkably, each endothelin receptor impacts DCs function in opposite manners. EDNRA expression in human DCs increases the expression of activation markers, enhancing DCs capability to activate T cells. EDNRA consequently mediates antigen presentation features of

DCs. DCs expressing EDNRA secrete immunomodulatory cytokine IL-12 and show reduced apoptosis. In contrast, expression of EDNRB by DCs decreases their maturation and survival suggesting that EDNRB suppresses immune response (Guruli *et al.*, 2004).

Several studies using tumor types other than melanoma have explored the microenvironmental role of endothelin signaling. Edn3/Ednrb is considered a marker for melanoma progression. EDN3 (Tang *et al.*, 2008) as well as EDNRB (Demunter *et al.*, 2001) expression has been detected in melanoma patients. Still, the exact cellular type expressing the receptor in the TME has not been determined. The present study investigated the microenvironmental expression of Ednrb in mouse and human melanomas.

2.2. Results

To better understand the role of endothelin signaling in the melanoma microenvironment green fluorescent protein (GFP) labeled YUMM1.7 and B16F10 murine melanoma cells were subcutaneously and intradermally injected in wild-type and *K5-tTA; TRE-Edn3-lacZ* (*K5-Edn3*, for simplicity) transgenic mice. The *K5-Edn3* mouse model was developed in our laboratory and overexpresses Edn3 in the skin. In this system, the overexpression of Edn3 is under the control of the keratin 5 promoter. Due to the abundance of Edn3 in the skin, melanocytes are maintained in the dermal/epidermal junction, mimicking their location in human skin (Garcia *et al.*, 2008). The aberrant expression of Edn3 in the skin of *K5-Edn3* mice is shown in Figure 2.2A.

Human melanoma initiation and development are mainly driven by activation of oncogenic protein BRAF^{V600E} as well as deletion of PTEN and CDKN2A genes (Shain and Bastian, 2016). These genetic alterations have recently been modeled in the YUMM1.7 mouse cell line (Meeth *et al.*, 2016). YUMM1.7 cells were derived from *BRaf*^{V600E/+}; *Pten*^{-/-}; *Cdkn2a*^{-/-} transgenic mice (Scortegagna *et al.*, 2014). Consequently, these cells carry mutations commonly found in human melanomas. Since YUMM1.7 cells are not pigmented they were labeled with GFP to facilitate the detection of metastatic cells. Figure 2.2B illustrates the protocol used to generate the YUMM1.7-GFP melanoma cells. B16F10 melanoma cells were derived from a spontaneous murine tumor in the early 1970's (Fidler, 1973) and are still extensively employed in cancer research due to its highly aggressive features. B16F10-GFP cells were injected in both *K5-Edn3* and wild-type mice to test the environmental role of Edn3 in a widely used melanoma model.

2.2.1. Endothelin increases tumor aggressiveness

YUMM1.7-GFP melanoma cells generated detectable tumors 7 days after subcutaneous injection. Both wild-type and *K5-Edn3* tumors had similar growth rates during the following 7 days. Seventeen days after injection the subcutaneous tumors in *K5-Edn3* mice started to grow faster than wild-type. *K5-Edn3* and wild type tumors had an average volume of 1054 mm³ and 673 mm³, respectively. *K5-Edn3* tumors continued to grow faster for the next days. Twenty-one days after injection *K5-Edn3* tumors were significantly larger ($p < 0.05$) than wild-type tumors (Figure 2.3A). Once tumors reached a volume close to 2 cm³ the mice were euthanized. Autopsy did not reveal any visible metastasis although cross-

sectioning of lungs showed the presence of GFP⁺ cells in the lungs of one *K5-Edn3* mouse (Figure 2.3B). Since Edn3 overexpression in the *K5-Edn3* mice is restricted to the skin, YUMM1.7-GFP cells were intradermally injected in the mice to ensure that melanoma cells were exposed to an Edn3-rich environment. Intradermal injection of YUMM1.7-GFP cells showed similar results to those observed upon subcutaneous injection. Intradermal YUMM1.7-GFP tumors generated in *K5-Edn3* mice were approximately two times larger than in wild-type mice ($p < 0.05$) (Figure 2.4A) and lung metastases ($n=1$) was detected only when the primary tumors were exposed to abundant Edn3 (Figure 2.4B).

In order to validate the observations made using YUMM1.7-GFP cells, B16F10-GFP melanoma cells were injected in both wild-type and *K5-Edn3* mice. Similar to YUMM1.7-GFP, intradermal B16F10-GFP tumors were more aggressive when growing in presence of Edn3. Tumors generated in *K5-Edn3* mice were six times larger than in wild-type mice ($p < 0.05$) (Figure 2.5A) and only *K5-Edn3* tumor ($n=1$) metastasized to the lungs (Figure 2.5B). These results indicate that Edn3 is responsible for melanoma growth and corroborates previous reports claiming that endothelin is considered a marker for melanoma progression.

2.2.2. Melanoma cells express Ednrb

Given the effect of overexpression of Edn3 on YUMM1.7-GFP tumors, I asked whether tumor cells express Ednrb and therefore are capable to respond to Edn3 present in the microenvironment. EDNRB expression has already been demonstrated in human melanoma cell lines. Immunofluorescence staining for

GFP and Ednrb detected GFP⁺ melanoma cells expressing Ednrb in both wild-type and *K5-Edn3* tumors (Figure 2.6A, white arrows). The antibody against GFP was used in order to amplify the signal for immunostaining. The visual inspection of four sections per tumor from YUMM1.7-GFP tumors (n=5/group) showed that very few melanoma cells expressed Ednrb.

In an effort to quantify Ednrb⁺/GFP⁺ melanoma cells, tumors were dissociated and analyzed using flow cytometry. A small population of GFP⁺ melanoma cells that expressed Ednrb was found in both groups (Figure 2.6B). There was no significant difference ($p>0.05$) between the amount of Ednrb⁺/GFP⁺ melanoma cells in wild-type and experimental tumors (Figure 2.6C). Immunostaining showed that a large number of GFP⁻ cells were positive for Ednrb (Figure 2.6A). Additionally, flow cytometry plots revealed a large and well-defined population of GFP⁻/Ednrb⁺ cells (Figure 2.6B). These observations suggest that Ednrb is expressed in stromal cells.

2.2.3. Ednrb expression in the melanoma microenvironment

To further characterize the populations of non-tumorigenic cells present in the TME, tumors were stained with different markers of stromal cells and analyzed using flow cytometry. Fibroblasts were detected using CD140a (PDGF-R) marker (Betsholtz, Karlsson and Lindahl, 2001), endothelial cells were labeled with CD146 antibody (Bardin *et al.*, 2001) and leukocytes were identified using the pan-leukocyte marker CD45 (Trowbridge and Thomas, 1994). The presence of immune cells from the myeloid lineage (Misharin *et al.*, 2013) were assessed using the

following markers: F4.80 for macrophages (Austyn and Gordon, 1981), Ly6G for neutrophils (Daley *et al.*, 2007) and CD11c for DCs (Osugi, Vuckovic and Hart, 2002). Lastly, cells from the lymphoid lineage, namely natural killer (NK) cells, CTLs, and Tregs were detected using NK1.1 (Stenström *et al.*, 2005), CD8a (Bierer *et al.*, 1989) and CD25 (IL-2R α) (Lee, 2017) markers, respectively.

All markers used stained positively demonstrating that the YUMM1.7-GFP melanoma microenvironment is very complex and comprised of a mixture of stromal cells (Figure 2.7). Overall, the number of non-malignant cells in tumors resulting from subcutaneous injections in wild-type and *K5-Edn3* tumors was not significantly different ($p>0.05$). The same pattern was observed in intradermal tumors ($p>0.05$) (Figure 2.7). One crucial exception was observed for Tregs. In both subcutaneous and intradermal injection-generated melanomas there were approximately two times more Tregs in *K5-Edn3* tumors when compared to wild-type tumors ($p<0.01$) (Figure 2.7C). This finding indicates that when Edn3 is present in the melanoma microenvironment there is an enrichment of intratumoral Tregs.

Immunofluorescence staining for Ednrb and the pan-leukocytes marker CD45 revealed that in both wild-type and experimental tumors there were several immune cells that expressed Ednrb (Figure 2.8). To further characterize the immune cell populations that expressed Ednrb, tumors were again dissociated and stained for flow cytometry analysis using different markers for stromal cells and co-stained with Ednrb antibody. All assessed stromal cells expressed Ednrb (Figure

2.9) but the exact populations of cells varied depending on the tumor location. Subcutaneous tumors in *K5-Edn3* mice exhibited more Ednrb⁺ endothelial cells (Figure 2.9A) and CTLs (Figure 2.9C) than wild-type tumors ($p < 0.05$), a pattern not replicated in intradermal tumors ($p > 0.05$). On the other hand, intradermal tumors in *K5-Edn3* mice showed more DCs (Figure 2.9B) that expressed Ednrb ($p < 0.05$), a trend not observed in subcutaneous tumors ($p > 0.05$). Treg was the only population that consistently expressed high levels of Ednrb in both subcutaneous and intradermal tumors in *K5-Edn3* mice ($p < 0.05$) (Figure 2.9C). This result corroborates the previous finding that the endothelin axis affects intratumoral Tregs.

2.2.4. Ednrb expression in human melanoma

Lastly, I investigated whether the findings from YUMM1.7-GFP melanoma mouse model are relevant for melanoma patients. Publicly available RNA-seq data of melanoma samples from 19 patients (Tirosh *et al.*, 2016) showed that EDNRB is expressed by melanoma cells and several populations of stromal cells in the TME (Figure 2.10A). In contrast to YUMM1.7-GFP tumors, malignant cells in the human melanoma TME highly expressed EDNRB. As noticed in the YUMM1.7-GFP tumors, fibroblasts, endothelial cells, macrophages, T, and NK cells expressed different levels of EDNRB. T cells exhibited the highest level of EDNRB expression in melanoma microenvironment. Next, the expression of EDNRB in intratumoral Tregs was assessed. Tregs were defined as the subpopulation of T cells expressing CD25 or FOXP3. There were few intratumoral Tregs that expressed EDNRB in melanoma tumors (Figure 2.10B). These data are in

accordance with the findings in the YUMM1.7-GFP tumors and suggest that endothelin signaling is important for stromal cells in the melanoma microenvironment, particularly Tregs.

2.3. Discussion

Notwithstanding initial controversy (Kikuchi *et al.*, 1996), *Ednrb* has long been demonstrated to be overexpressed in aggressive melanomas. Indeed, *Edn3/Ednrb* signaling is currently considered a marker for melanoma progression (Demunter *et al.*, 2001). The present study strengthens these earlier findings showing that overexpression of *Edn3* in the melanoma microenvironment increases tumor aggressiveness. Larger and metastatic melanoma were developed when primary tumors were exposed to a microenvironment rich in *Edn3*. Similar results were observed using YUMM1.7 cell line, which harbor *Braf*^{V600E} (Meeth *et al.*, 2016), and B16F10, a melanoma cell line considered *Braf* wild-type (Melnikova *et al.*, 2004; Hooijkaas *et al.*, 2012). Oncogenic BRAF^{V600E} is found in about 50% of human melanomas (TCGA, 2015). Findings from the present study suggest that endothelin signaling is relevant to a broad range of melanomas, including BRAF^{V600E} and BRAF wild-type. It should be noted that endothelin might be crucial for BRAF^{V600E} mutated melanomas. Recent reports have shown that melanoma treated with BRAFi tend to have increased expression of both *Ednrb* and *Edn1* (Asundi *et al.*, 2014; Smith *et al.*, 2017). Thus, combinatory treatments targeting endothelin signaling and oncogenic BRAF might be an effective therapeutic approach for a particular subset of melanoma patients.

Despite the canonical role of Edn3/Ednrb axis during melanoma progression, to this date it is not clear the cellular distribution of endothelin signaling components in the melanoma microenvironment. *In vitro* and *in vivo* expression of EDN1 (Chiriboga *et al.*, 2016) as well as EDN3 (Tang *et al.*, 2008) have been described in human melanoma cells. Recently, an *in vitro* study showed that EDN1 mediates the communication between endothelial and melanoma cells and enhances their vasculogenic ability (Spinella *et al.*, 2014). Expression of EDN1 was also detected in intratumoral macrophages from melanoma biopsies (Chiriboga *et al.*, 2016). EDNRB expression in melanoma has been mostly described in melanoma cell lines (Yohn *et al.*, 1994; Lahav, Heffner and Patterson, 1999; Bagnato *et al.*, 2004; Asundi *et al.*, 2011). Although EDNRB expression is reported in patients with melanoma (Bittner *et al.*, 2000; Demunter *et al.*, 2001), the precise cellular type expressing the receptor in the TME had not been addressed. In this respect, Ednrb expression was detected in GFP⁺ melanomas cells from YUMM1.7-GFP tumors. However, no difference in the amount of GFP⁺/Ednrb⁺ was found when *K5-Edn3* and wild-type tumors were compared. Yet, in both control and experimental YUMM1.7-GFP tumors the expression of Ednrb was detected in a small percentage of melanoma cells. Many non-malignant cells expressed Ednrb in YUMM1.7-GFP tumors, suggesting a possible environmental role of endothelin signaling in the melanoma microenvironment. Thus, stromal activation of endothelin signaling might be central to the aggressive phenotype of *K5-Edn3* tumors. In breast cancer, the role of stromal Ednrb has been explored in detail. Injection of breast cancer cells overexpressing Ednra generated tumors with

diminished growth and metastatic potential in *Ednrb* deficient rat. Lack of stromal *Ednrb* did not affect angiogenesis but considerably impaired the infiltration of immune cells and cytokine production resulting in reduced tumor aggressiveness. The absence of *Ednrb* in the TME decreased the recruitment of TAMs and the amount of pro-inflammatory cytokine TNF- α . In contrast, the amounts of T lymphocytes and NK cells, as well as IL-10 cytokine were not affected by the deficiency of *Ednrb* in the TME (Binder et al., 2009).

Melanoma microenvironment is comprised of several cell types. Tirosh and colleagues elegantly described different populations of stromal cells existing in human metastatic melanoma microenvironment. CAFs and endothelial cells together with NK and macrophages represent a small percentage of melanoma stroma. The majority of melanoma stroma consists of B lymphocytes and T cell subtypes including CD8, CD4, and Treg (Tirosh *et al.*, 2016). Basic characterization of YUMM1.7 tumors revealed that these melanomas have considerable immune infiltrate. As opposed to human melanomas the majority of immune infiltrate was comprised of macrophages. Lymphocytes represented less than 1% of immune cells in the microenvironment (Meeth *et al.*, 2016). A more sophisticated portrayal of the melanoma immune infiltrate from a different *Braf*^{V600E} mutated mouse melanoma cell line (BPD6) showed the presence CTLs, DCs, TAMs and myeloid-derived suppressor cells (MDSCs) in the TME (Liu *et al.*, 2018).

The above-mentioned findings were confirmed in this study by demonstrating that YUMM1.7-GFP melanoma microenvironment is comprised of CAFs, endothelial cells and a variety of leukocytes including macrophages, neutrophils, DCs, NK, CTLs and Tregs. The higher amount of T cells in YUMM1.7-GFP tumors compared to the original YUMM1.7 cells might be explained by differences in the genetic background of mice used in the present study. In order to make the YUMM1.7 cells syngeneic (that is, genetically identical) to C57BL/6 mice, the YUMM1.7 cells were generated in transgenic mice after a series of backcrosses with C57BL/6 mice (Meeth *et al.*, 2016). In contrast, the *K5-Edn3* transgenic mice and their wild-type counterparts were generated using mice from C57BL/6 and FVB/N genetic backgrounds (Garcia *et al.*, 2008). Therefore, the YUMM1.7-GFP cells might not be syngeneic to the *K5-Edn3* mice. Upon injection in immunocompetent *K5-Edn3* mice, YUMM1.7-GFP cells might have induced a strong immune response, which may explain the discrepancy in the T cell infiltrates of YUMM1.7 and YUMM1.7-GFP tumors. Another reason could be the fact that YUMM1.7-GFP cells were GFP labeled using lentivirus. GFP is a protein originated from jellyfish that can induce immune responses in immunocompetent mice (Day *et al.*, 2014), a reaction also triggered by viral particles of lentivirus vector (Nayak and Herzog, 2010).

The present study showed for the first time that *Ednrb* is expressed in several cell populations within melanoma microenvironment. The microenvironmental role of endothelin signaling has been explored in many tumor types other than melanoma. Endothelin derived from colorectal cancer cells acts in a paracrine

fashion on endothelin receptors exhibited by fibroblast and stimulates its growth, migration, and contraction. Blockage of both EDNRA and EDNRB efficiently inhibited the EDN1 induced effects in colonic fibroblasts (Haque et al., 2013). In response to EDN1, CAFs reduce the expression of genes associated with inflammation, namely IL-8 and SMAD5 (Knowles et al., 2012). This suggests that, although considered a pro-inflammatory cytokine (Nett *et al.*, 2006), in some contexts endothelin might negatively regulate the inflammatory milieu in the TME.

Stromal endothelin is classically associated with angiogenesis. Indeed, high expression of EDN1 is correlated with neovascularization in ovarian cancer (Salani, Castro, *et al.*, 2000). A mechanism employed by mesenchymal stem cells (MSC) that contributes to endothelin-induced angiogenesis in the TME has recently been described. MSCs produce IL-6, which controls the secretion of EDN1 by colorectal cancer cells. Endothelin promotes ERK and Akt phosphorylation in endothelial cells, thus increasing endothelial cells recruitment and formation of new blood vessels (Huang *et al.*, 2013). Transcriptional profile of endothelial cells from ovarian cancer revealed that more aggressive tumors have less tumor infiltrates lymphocytes (TIL) because of EDNRB overexpression. EDNRB decreases the expression of adhesion molecule ICAM-1 and consequently prevents recruitment of CTLs to the TME. Endothelin attenuates lymphocyte clustering to endothelial cells even in the presence of pro-inflammatory cytokine TNF- α . Therefore, EDNRB activation in endothelial cells present in the TME creates an endothelial barrier sustaining the immune-privileged microenvironment. Preclinical studies showed that EDNRB blockage enhances otherwise ineffective immunotherapy strategies

increasing CD8⁺ homing to the TME. Targeting EDNRB also promotes activation of anti-tumor lymphocytes as shown by the upregulation of IFN- γ and IL-2 cytokines (Buckanovich et al., 2008). Thus, EDNRB activation in the TME suppresses T cell recruitment facilitating immune escape and EDNRB antagonists can be used in combination with immunotherapy to improve anti-tumor immune response (Kandalaft *et al.*, 2009, 2011). EDNRA seems to increase ICAM-1 expression on endothelial cells enhancing the recruitment of tumor inhibitory immune cells (Coffman et al., 2013). Hence, EDNRA activation in the TME is important for anti-tumor response while EDNRB stimulation prevents immune response against tumor cells (Aubert and Juillerat-Jeanneret, 2016). This data is to a certain extent conflicting with the observation that subcutaneous tumors in *K5-Edn3* mice had great quantities of endothelial cells that express *Ednrb* while exhibiting more *Ednrb*⁺ CTL. The fact that *K5-Edn3* tumors were larger and metastatic suggests that the effect of *Ednrb* in subcutaneous YUMM1.7-GFP tumors is not restricted to the recruitment of CTLs. This effect might be related to other stromal population such as Tregs, which can further inhibit tumor-specific CTLs.

Endothelin signaling is an important regulator of DCs function and survival. Activation of immature DCs with TNF- α induce human DCs to secrete endothelin and to express endothelin receptors. EDNRA stimulation enhances antigen presentation by DCs as well as production of IL-12, a major regulator of T_H1 polarization. On the other hand, activation of EDNRB prevents DCs activation. These data suggest that endothelin might have an autocrine effect on DCs and the

balance of endothelin receptors is involved in the generation of T_H1 response (Guruli *et al.*, 2004). The negative regulatory effect of Ednrb in anti-tumorigenic cells such as DCs might explain the fact that *K5-Edn3* tumors are more aggressive even though they had a higher number of Ednrb⁺ DCs.

The present study supports the hypothesis that endothelin signaling may have multiple effects in melanoma microenvironment given that different populations of stromal cells expressed Ednrb in the YUMM1.7-GFP tumors. Importantly, this is the first report of Ednrb expression in NK cells and Tregs in TME. In both control and experimental YUMM1.7-GFP melanomas, Ednrb expression was detected in anti-tumor cells including CTLs, NK and DCs as well as pro-tumorigenic cells, namely CAFs, TAMs, and Tregs. The fact that Ednrb expression is detected in both anti- and pro-tumorigenic cells suggests potential opposite functions of endothelin signaling in the melanoma microenvironment. Thus, the balance of endothelin signaling activation among stromal cells that antagonize or promote tumor development determines the fate of melanoma progression. Given that *K5-Edn3* tumors have a more aggressive phenotype, it is reasonable to assert that in Edn3-rich microenvironment the net result favors the effect of endothelin in pro-tumorigenic cells.

Treg is the only population that is numerically different when *K5-Edn3* tumors are compared to wild-type tumors. Therefore, it is tempting to claim that Tregs are responsible for creating an immunosuppressive environment inside melanoma tumors, allowing for the escape from immunosurveillance. Aggressive

glioblastomas express high levels of EDNRB and show reduced infiltration of CTL along with increased number of Tregs. This data supports the notion that activation of stromal EDNRB suppresses host anti-tumor immunity (Nakashima *et al.*, 2016). It is not clear whether the augmented amount of intratumoral Ednrb⁺ Tregs in the *K5-Edn3* tumors is a consequence of increased Treg recruitment, proliferation or differentiation from conventional T cells. Finally, EDNRB⁺ Tregs were detected in the human metastatic melanoma microenvironment suggesting that Tregs might respond to EDN1 and EDN3 present in human melanomas and orchestrate immunosuppression accountable for melanoma aggressiveness. Together, the results presented here support the use of EDNRB blockage in combination with immunotherapy (Buckanovich *et al.*, 2008) as a therapeutic approach to be considered for patients with aggressive melanomas.

2.4. Methods

2.4.1. Mice and genotyping

The *K5-tTA; TRE-Edn3-lacZ* (*K5-Edn3*, for simplicity) transgenic mice were generated in our laboratory (Garcia *et al.*, 2008). *K5-Edn3* mice constitutively overexpress Edn3 in the skin using a tetracycline-inducible system. Transgenic expression of Edn3 is driven by the tTA activator in the regulatory region of the *Bos taurus* gene for Keratin 5. Mice from *K5-tTA; TRE-Edn3-lacZ* intercrosses that harbored either *K5-tTA* or *TRE-Edn3-lacZ* transgene were denominated wild-type due the lack of transgenic overexpression of Edn3. A small piece of mouse tail was used as a DNA source for genotyping. After DNA extraction PCR was carried out

using the following primers: 5'-CCAGGTGGAGTCACAGGATT-3' and 5'-ACAGAGACTGTGGACCACCC-3' for *K5-tTA* transgene and 5'-AGGCCTGTGCACACTTCTGT-3' and 5'-TCCTTGTGAAACTGGAGCCT-3' for the *TRE-Edn3-lacZ* transgene. All animals were housed in the University Animal Care Facility at Florida International University. The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Florida International University protocol number 16-018.

2.4.2. Cell lines

YUMM1.7, a gift from Dr. Marcus Bosenberg (Yale University), and B16F10 murine melanoma cell (ATCC) lines were labeled with GFP using recombinant lentivirus pHIV-Ednrb-EGFP. The lentivirus transduction procedure was carried out at Florida International University Tissue Cell Culture Facility. The generation of GFP labeled cells was approved by Institutional Biosafety Committee (IBC) protocol number 16-027. YUMM1.7-GFP cells were cultured with DMEM/F-12 supplemented with 10% fetal bovine serum and penicillin/streptomycin. B16F10-GFP cells were cultured in supplemented DMEM medium.

2.4.3. Tumor cell injection

A total of 100,000 melanoma cells were injected in the shaved flank of *K5-Edn3* or wild-type mice. For subcutaneous injection cells were resuspended into 100µl of PBS and injected under the skin using 25G needle. For intradermal injection cells were resuspended into 50µl of PBS and injected under the superficial layer of epidermis using 30G needle (Shimizu, 2004). Tumor length and width were

measured twice a week using a digital caliper. Tumor volume was calculated using the following formula: $\text{volume} = (\text{length} \times \text{width} \times \text{width})/2$ (Cintolo *et al.*, 2016). Mice were euthanized when tumors reached 2 cm³ of volume. Statistical analysis was carried out with Student T-Test and considered to be significantly different at $p < 0.05$.

2.4.4. Flow Cytometry

Harvested tumors were minced finely with a scalpel blade and dissociated using collagenase/dispase (Roche 10 269 638) for 45min followed by digestion with trypsin 0.25% at 37°C for 5min. Cell suspension was filtered using 40µM cell strainer and red blood cells were lysed with ACK lysis buffer. Single-cell suspension was stained at 4°C for 30min using the following phycoerythrin-conjugated antibodies: CD140 (0.25µg, Biolegend, 135905), CD146 (0.06µg, Biolegend, 134703), CD45 (0.25µg, Biolegend, 103105), F4/80 (1µg, eBiosciences, 12-4801-82), Ly6G (0.25µg, Biolegend, 127607), CD11c (0.25µg, Biolegend, 117307), NK1.1 (0.25µg, Biolegend, 108707), CD8a (0.25µg, Biolegend, 100707) and CD25 (1µg, Biolegend, 102007). Single-cell suspension was also stained with unconjugated rabbit Ednrb antibody (1:200, Proteintech, 20964-1-AP) followed by secondary staining using anti-rabbit PerCP/Cy5.5 antibody (1µg, Santa Cruz, sc-45109) or anti-rabbit Alexa 647 antibody (1:2000, Abcam, ab150075). Fifty thousand events were acquired using Accuri C6 (BD Biosciences) flow cytometer. The flow cytometric profile of each sample was analyzed using FlowJo (TreeStar) software. Statistical analysis was carried out with Student T-Test and considered to be significantly different at $p < 0.05$.

2.4.5. Histological and Immunofluorescence Analysis

Tissues were fixed overnight with 4% paraformaldehyde (PFA), sequentially incubated for 12h with 10% and 20% sucrose and cryo-embedded in OCT (Fisher 23-730-571). Cryosections (10 μ M) were incubated with blocking buffer (10% normal serum, 0.3% Triton X-100, 1% BSA in 1x PBS) at room temperature for 1h. Next, primary antibodies were diluted in dilution buffer (1% normal serum, 0.3% Triton X-100, 1% BSA in 1x PBS) and added to cryosections for incubation at room temperature for 1 hour or at 4°C overnight. Primary antibodies used: Edn3 (1:200, Proteintech, 10674-1-AP), CD45 (1:200, Biolegend, 103101), Ednrb (1:400, Proteintech, 20964-1-AP) and GFP (1:500, Aves Labs, GFP-1020). Antibody against GFP was used in order to amplify the signal for immunostaining. Where necessary, GFP fluorescence from lentivirus expression was photobleached by treatment with 3% H₂O₂ in methanol and illumination on a fluorescent light box for 4 hours. After 3 sequential washes with 1xPBS, cryosections were incubated with secondary antibody for 1 hour at room temperature. Secondary antibodies used: anti-rabbit Alexa 488 (1:200, Invitrogen, A11008), anti-chicken Alexa 488 (1:500, Abcam, ab150169), anti-rat Alexa 488 (1:200, Abcam, ab150159), anti-rabbit Alexa 594 (1:200, Invitrogen, A11012). Then, slides were mounted with mounting medium containing DAPI (Abcam, ab104139). Slides were visualized using Leica DMRB fluorescent microscope and digital pictures were captured with a Leica DFC450C camera.

2.4.6. Publicly available RNA-seq data of melanoma samples

Single-cell gene expression data of melanoma samples was obtained from GEO repository, access number GSE72056. Cell types were defined according to the classification from Tirosh, *et al.* (2016). Treg cells were defined as the subpopulation of T cells that have the expression level of CD25 or FOXP3 ≥ 0.5 FPKM.

2.5. Figures

Figure 2.1 Mechanisms of action of endothelin in the TME

Endothelin signaling is activated in diverse populations of stromal cells in the TME. Activation of endothelin receptors promotes growth and migration of fibroblasts as well as production of ECM components and proteases. Ednrb activation on endothelial cells stimulates angiogenic events such as proliferation and migration of endothelial cells. Endothelin also controls the production of angiogenic factor VEGF. The differential expression of endothelin receptors in endothelial cells regulates the expression of adhesion molecules that eventually control the recruitment of lymphocytes to the TME. The functions of APCs, namely DCs and macrophages, are also under the control of endothelin signaling.

Figure 2.2 Melanoma cell line and mouse model

A. Wild-Type mouse and transgenic mouse model *K5-Edn3* used for subcutaneous and intradermal injection of established YUMM1.7-GFP melanoma cells. **B.** Schematic protocol used to generate YUMM1.7-GFP melanoma cell line as described in (Meeth *et al.*, 2016).

A**YUMM1.7-GFP subcutaneous** **B**

Figure 2.3 Endothelin overexpression increases aggressiveness of subcutaneous YUMM1.7-GFP melanoma tumors

A. Tumor volume of mice that received subcutaneous (n=8) injections of YUMM1.7-GFP melanoma cells. **B.** GFP⁺ cells (green) in lungs of wild-type and *K5-Edn3* mice subcutaneously injected with YUMM1.7-GFP cells. * $p < 0.05$

A**YUMM1.7-GFP intradermal** **B**

Wild-Type

K5-Edn3

Figure 2.4 Endothelin overexpression increases aggressiveness of intradermal YUMM1.7-GFP melanoma tumors

A. Tumor volume of mice that received intradermal (n=13) injections of YUMM1.7-GFP melanoma cells. **B.** GFP⁺ cells (green) in lungs of wild-type and *K5-Edn3* mice intradermally injected with YUMM1.7-GFP cells. **p*<0.05

A**B16F10-GFP intradermal** **B**

Wild-Type

K5-Edn3

Figure 2.5 Endothelin overexpression increases aggressiveness of intradermal B16F10-GFP melanoma tumors

A. Tumor volume of mice that received intradermal (n=11) injections of B16F10-GFP melanoma cells. **B.** GFP⁺ cells (green) in lungs of wild-type and *K5-Edn3* mice intradermally injected with B16F10-GFP cells. * $p < 0.05$

Figure 2.6 Ednrb is expressed in melanoma cells

A. Representative photomicrographs of immunofluorescence staining of cryosections of intradermally injected YUMM1.7-GFP tumors in wild-type and *K5-Edn3* mice for GFP and Ednrb. White arrow showing double positive cells. **B.** Flow Cytometry plots of GFP⁺/Ednrb⁺ melanoma cells of subcutaneous and intradermal YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice. **C.** Percent quantification of flow cytometry data (n=3/group). **p*<0.05.

Figure 2.7 Populations of stromal cells present in the melanoma TME

Percent quantification of mesenchymal cells (**A**), myeloid (**B**) and lymphoid (**C**) cells in subcutaneous and intradermal YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice (n=3/group). ** $p < 0.01$.

Figure 2.8 Ednrb is expressed by stromal cells in the melanoma TME

Representative photomicrographs of immunofluorescence staining of cryosections of intradermally injected YUMM1.7-GFP tumors in wild-type (**A**) and *K5-Edn3* mice (**B**) for CD45 (green) and Ednrb (red). White arrows showing double positive cells.

Figure 2.9 Ednrbl is expressed by a variety of cell types in the melanoma TME

Percent quantification of Ednrbl⁺ mesenchymal cells (A), myeloid (B) and lymphoid (C) in subcutaneous and intradermal YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice (n=3/group) **p*<0.05, ***p*<0.01.

Figure 2.10 EDNRB is expressed by a variety of cell types within the human melanoma TME

A. Violin plots showing frequency of malignant and stromal cells expressing different EDNRB levels from the single-cell RNA sequencing data of melanoma samples from 19 patients (Tirosh *et al.*, 2016). **B.** Violin plot of EDNRB expression in Tregs cells. Treg cells were defined as the subpopulation of T cells that have the expression level of CD25 or FOXP3 ≥ 0.5 FPKM. Pre-processed RNA sequencing data were downloaded from supplementary material.

2.6. References

- Asundi, J. *et al.* (2011) 'An Antibody-Drug Conjugate Targeting the Endothelin B Receptor for the Treatment of Melanoma', *Clinical Cancer Research*, 17(5), pp. 965–975. doi: 10.1158/1078-0432.CCR-10-2340.
- Asundi, J. *et al.* (2014) 'MAPK pathway inhibition enhances the efficacy of an anti-endothelin B receptor drug conjugate by inducing target expression in melanoma.', *Molecular cancer therapeutics*, 13(6), pp. 1599–1610. doi: 10.1158/1535-7163.MCT-13-0446.
- Aubert, J. D. and Juillerat-Jeanneret, L. (2016) 'Endothelin-Receptor Antagonists beyond Pulmonary Arterial Hypertension: Cancer and Fibrosis', *Journal of Medicinal Chemistry*, 59(18), pp. 8168–8188. doi: 10.1021/acs.jmedchem.5b01781.
- Austyn, J. M. and Gordon, S. (1981) 'F4/80, a monoclonal antibody directed specifically against the mouse macrophage', *European Journal of Immunology*, 11(10), pp. 805–815. doi: 10.1002/eji.1830111013.
- Bagnato, A. *et al.* (2004) 'Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions in Melanoma Cell Progression', *Cancer research*, 64(4), pp. 1436–1443.
- Bardin, N. *et al.* (2001) 'Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion', *Blood*, 98(13), pp. 3677–3684. doi: 10.1182/blood.V98.13.3677.
- Betsholtz, C., Karlsson, L. and Lindahl, P. (2001) 'Developmental roles of platelet-derived growth factors', *BioEssays*, 23(6), pp. 494–507. doi: 10.1002/bies.1069.
- Bierer, B. E. *et al.* (1989) 'The Biologic Roles of CD2, CD4, and CD8 in T-Cell Activation', *Annual Review of Immunology*, 7(1), pp. 579–599. doi: 10.1146/annurev.iy.07.040189.003051.
- Binder, C. *et al.* (2009) 'Stromal endothelin B receptor-deficiency inhibits breast cancer growth and metastasis', *Molecular Cancer Therapeutics*, 8(8), pp. 2452–2460. doi: 10.1158/1535-7163.MCT-09-0032.
- Bittner, M. *et al.* (2000) 'Molecular classification of cutaneous malignant melanoma by gene expression profiling.', *Nature*, 406(6795), pp. 536–540. doi: 10.1038/35020115.
- Braeuer, R. R. *et al.* (2014) 'Why is melanoma so metastatic?', *Pigment Cell & Melanoma Research*, 27(1), pp. 19–36. doi: 10.1111/pcmr.12172.
- Buckanovich, R. J. *et al.* (2008) 'Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy.', *Nature medicine*, 14(1), pp. 28–36. doi: 10.1038/nm1699.

- Chen, C. C. *et al.* (2014) 'The endothelin-integrin axis is involved in macrophage-induced breast cancer cell chemotactic interactions with endothelial cells', *Journal of Biological Chemistry*, 289(14), pp. 10029–10044. doi: 10.1074/jbc.M113.528406.
- Chiriboga, L. *et al.* (2016) 'Endothelin-1 in the tumor microenvironment correlates with melanoma invasion', *Melanoma Research*, 26(3), pp. 236–44. doi: 10.1097/CMR.0000000000000235.
- Cintolo, J. A. *et al.* (2016) 'Type I-polarized BRAF-pulsed dendritic cells induce antigen-specific CD8⁺ T cells that impact BRAF-mutant murine melanoma.', *Melanoma research*, 26(1), pp. 1–11. doi: 10.1097/CMR.0000000000000203.
- Coffman, L. *et al.* (2013) 'Endothelin receptor-A is required for the recruitment of antitumor T cells and modulates chemotherapy induction of cancer stem cells', *Cancer Biology and Therapy*, 14(2), pp. 184–192. doi: 10.4161/cbt.22959.
- Daley, J. M. *et al.* (2007) 'Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice', *Journal of Leukocyte Biology*, 83(1), pp. 64–70. doi: 10.1189/jlb.0407247.
- Day, C. P. *et al.* (2014) "Glowing head" mice: A genetic tool enabling reliable preclinical image-Based evaluation of cancers in immunocompetent allografts', *PLoS ONE*, 9(11), p. e109956. doi: 10.1371/journal.pone.0109956.
- Demunter, A. *et al.* (2001) 'Expression of the endothelin-B receptor in pigment cell lesions of the skin: Evidence for its role as tumor progression marker in malignant melanoma', *Virchows Archiv*, 438(5), pp. 485–491. doi: 10.1007/s004280000362.
- Egidy, G., Juillerat-Jeanneret, L., *et al.* (2000) 'Modulation of human colon tumor-stromal interactions by the endothelin system', *American Journal of Pathology*, 157(6), pp. 1863–1874. doi: 10.1016/S0002-9440(10)64825-0.
- Egidy, G., Eberl, L. P., *et al.* (2000) 'The endothelin system in human glioblastoma', *Laboratory Investigation*, 80(11), pp. 1681–1689. doi: 10.1038/labinvest.3780178.
- Fidler, I. J. (1973) 'Selection of successive tumour lines for metastasis.', *Nature: New biology*, 242(118), pp. 148–149.
- Garcia, R. J. *et al.* (2008) 'Endothelin 3 Induces Skin Pigmentation in a Keratin-Driven Inducible Mouse Model', *Journal of Investigative Dermatology*, 128(1), pp. 131–142. doi: 10.1038/sj.jid.5700948.
- Giaid, A. *et al.* (1990) 'Detection of endothelin immunoreactivity and mRNA in pulmonary tumours', *The Journal of Pathology*, 162(1), pp. 15–22. doi: 10.1002/path.1711620105.
- Gohji, K. *et al.* (2001) 'Expression of endothelin receptor A associated with prostate cancer progression', *Journal of Urology*, 165(3), pp. 1033–1036. doi: 10.1016/S0022-5347(05)66597-4.

- Grimshaw, M. J. (2005) 'Endothelins in breast tumour cell invasion', *Cancer Letters*, 222(2), pp. 129–138. doi: 10.1016/j.canlet.2004.08.029.
- Grimshaw, M. J., Wilson, J. L. and Balkwill, F. R. (2002) 'Endothelin-2 is a macrophage chemoattractant: Implications for macrophage distribution in tumors', *European Journal of Immunology*, 32(9), pp. 2393–2400. doi: 10.1002/1521-4141(200209)32:9<2393::AID-IMMU2393>3.0.CO;2-4.
- Guruli, G. *et al.* (2004) 'Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops', *Blood*, 104(7), pp. 2107–2115. doi: 10.1182/blood-2003-10-3559.
- Haque, S. -u. *et al.* (2013) 'Efficacy of the Specific Endothelin A Receptor Antagonist Zibotentan (ZD4054) in Colorectal Cancer: A Preclinical Study', *Molecular Cancer Therapeutics*, 12(8), pp. 1556–1567. doi: 10.1158/1535-7163.MCT-12-0975.
- Hooijkaas, A. I. *et al.* (2012) 'Targeting BRAF V600E in an inducible murine model of melanoma', *American Journal of Pathology*. Elsevier Inc., 181(3), pp. 785–794. doi: 10.1016/j.ajpath.2012.06.002.
- Huang, W.-H. *et al.* (2013) 'Mesenchymal stem cells promote growth and angiogenesis of tumors in mice', *Oncogene*. Nature Publishing Group, 32, pp. 4343–4354. doi: 10.1038/onc.2012.458.
- Kandalafi, L. E. *et al.* (2009) 'Endothelin B receptor, a new target in cancer immune therapy', *Clinical Cancer Research*, 15(14), pp. 4521–4528. doi: 10.1158/1078-0432.CCR-08-0543.
- Kandalafi, L. E. *et al.* (2011) 'Tumor immune surveillance and ovarian cancer', *Cancer and Metastasis Reviews*, 30(1), pp. 141–151. doi: 10.1007/s10555-011-9289-9.
- Kikuchi, K. *et al.* (1996) 'Decreased ET(B) receptor expression in human metastatic melanoma cells.', *Biochemical and biophysical research communications*, 219(3), pp. 734–739. doi: 10.1006/bbrc.1996.0303.
- Knowles, J. P. *et al.* (2012) 'Endothelin-1 stimulates colon cancer adjacent fibroblasts', *International Journal of Cancer*, 130(6), pp. 1264–1272. doi: 10.1002/ijc.26090.
- Lahav, R., Heffner, G. and Patterson, P. H. (1999) 'An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(20), pp. 11496–11500.
- Lee, G. R. (2017) 'Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells', *Mediators of inflammation*, 2017, pp. 1–9. doi: 10.1155/2017/5458178.

- Liu, Q. *et al.* (2018) 'Nanoparticle-Mediated Trapping of Wnt Family Member 5A in Tumor Microenvironments Enhances Immunotherapy for B-Raf Proto-Oncogene Mutant Melanoma', *ACS Nano*, 12(2), pp. 1250–1261. doi: 10.1021/acsnano.7b07384.
- Meeth, K. *et al.* (2016) 'The YUMM lines: a series of congenic mouse melanoma cell lines with defined genetic alterations', *Pigment Cell & Melanoma Research*, 29(5), pp. 590–7. doi: 10.1111/pcmr.12498.
- Melnikova, V. O. *et al.* (2004) 'Genomic alterations in spontaneous and carcinogen-induced murine melanoma cell lines.', *Oncogene*, 23(13), pp. 2347–2356. doi: 10.1038/sj.onc.1207405.
- Misharin, A. V. *et al.* (2013) 'Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung', *American Journal of Respiratory Cell and Molecular Biology*, 49(4), pp. 503–510. doi: 10.1165/rcmb.2013-0086MA.
- Nakashima, S. *et al.* (2016) 'Endothelin B receptor expression in malignant gliomas: the perivascular immune escape mechanism of gliomas', *Journal of Neuro-Oncology*. Springer US, 127(1), pp. 23–32. doi: 10.1007/s11060-015-2017-5.
- Nayak, S. and Herzog, R. W. (2010) 'Progress and prospects: Immune responses to viral vectors', *Gene Therapy*. Nature Publishing Group, 17(3), pp. 295–304. doi: 10.1038/gt.2009.148.
- Nelson, J. *et al.* (2003) 'The endothelin axis: Emerging role in cancer', *Nature Reviews Cancer*, 3(2), pp. 110–116. doi: 10.1038/nrc990.
- Nett, P. C. *et al.* (2006) 'Recent developments on endothelin antagonists as immunomodulatory drugs--from infection to transplantation medicine.', *Recent patents on cardiovascular drug discovery*, 1(3), pp. 265–276. doi: 10.2174/157489006778776990.
- Osugi, Y., Vuckovic, S. and Hart, D. N. J. (2002) 'Myeloid blood CD11C+ dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes', *Blood*, 100(8), pp. 2858–2866. doi: 10.1182/blood.V100.8.2858.
- Patel, K. V., Sheth, H. G. and Schrey, M. P. (1997) 'Stimulation or endothelin-1 secretion by human breast cancer cells through protein kinase A activation: A possible novel paracrine loop involving breast fibroblast-derived prostaglandin E2', *Molecular and Cellular Endocrinology*, 126(2), pp. 143–151. doi: 10.1016/S0303-7207(96)03983-4.
- Rosanò, L. and Bagnato, A. (2016) 'Endothelin therapeutics in cancer : Where are we ?', *American journal of physiology.*, 310(6). doi: 10.1152/ajpregu.00532.2015.

Rosanò, L., Spinella, F. and Bagnato, A. (2010) 'The importance of endothelin axis in initiation, progression, and therapy of ovarian cancer.', *American journal of physiology. Regulatory, integrative and comparative physiology*, 299(June 2010), pp. R395–R404. doi: 10.1152/ajpregu.00304.2010.

Rosanò, L., Spinella, F. and Bagnato, A. (2013) 'Endothelin 1 in cancer: biological implications and therapeutic opportunities.', *Nature reviews. Cancer*. Nature Publishing Group, 13(9), pp. 1–15. doi: 10.1038/nrc3546.

Said, N. *et al.* (2011) 'Tumor endothelin-1 enhances metastatic colonization of the lung in mouse xenograft models of bladder cancer.', *The Journal of clinical investigation*, 121(1), pp. 132–47. doi: 10.1172/JCI42912.

Salani, D., Taraboletti, G., *et al.* (2000) 'Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo', *American Journal of Pathology*, 157(5), pp. 1703–1711. doi: 10.1016/S0002-9440(10)64807-9.

Salani, D., Castro, V. Di, *et al.* (2000) 'Role of endothelin-1 in neovascularization of ovarian carcinoma', *American Journal of Pathology*, 157(5), pp. 1537–1547. doi: 10.1016/S0002-9440(10)64791-8.

Scortegagna, M. *et al.* (2014) 'Genetic inactivation or pharmacological inhibition of Pdk1 delays development and inhibits metastasis of Braf(V600E)::Pten(-/-) melanoma.', *Oncogene*, 33(34), pp. 4330–9. doi: 10.1038/onc.2013.383.

Shain, A. H. and Bastian, B. C. (2016) 'From melanocytes to melanomas', *Nat Rev Cancer*. Nature Publishing Group, 16(6), pp. 345–358. doi: 10.1038/nrc.2016.37.

Shimizu, S. (2004) 'Routes of Administration', in Hedrich, H. J. and Bullock, G. (eds) *The Laboratory Mouse*. Elsevier, pp. 527–543. doi: 10.1016/B978-0-12-336425-8.X5051-1.

Smith, M. P. *et al.* (2017) 'Targeting endothelin receptor signalling overcomes heterogeneity driven therapy failure', *EMBO Molecular Medicine*, 9(8), pp. 1011–1029. doi: 10.15252/emmm.

Spinella, F. *et al.* (2014) 'The interplay between hypoxia, endothelial and melanoma cells regulates vascularization and cell motility through endothelin-1 and vascular endothelial growth factor', *Carcinogenesis*, 35(4), pp. 840–848. doi: 10.1093/carcin/bgu018.

Stenström, M. *et al.* (2005) 'Natural killer T-cell populations in C57BL/6 and NK1.1 congenic BALB.NK mice - A novel thymic subset defined in BALB.NK mice', *Immunology*, 114(3), pp. 336–345. doi: 10.1111/j.1365-2567.2004.02111.x.

Tang, L. *et al.* (2008) 'Endothelin-3 is produced by metastatic melanoma cells and promotes melanoma cell survival', *Journal of Cutaneous Medicine and Surgery*, 12(2), pp. 64–70. doi: 10.2310/7750.2008.06164.

TCGA (2015) 'Genomic Classification of Cutaneous Melanoma', *Cell*, 161(7), pp. 1681–1696. doi: 10.1016/j.cell.2015.05.044.

Tirosh, I. *et al.* (2016) 'Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq.', *Science*, 352(6282), pp. 189–96. doi: 10.1126/science.aad0501.

Trowbridge, I. S. and Thomas, M. L. (1994) 'CD45: An Emerging Role as a Protein Tyrosine Phosphatase Required for Lymphocyte Activation and Development', *Annual Review of Immunology*, 12(1), pp. 85–116. doi: 10.1146/annurev.iy.12.040194.000505.

Yohn, J. J. *et al.* (1994) 'Human Melanoma Cells Express Functional Endothelin-1 Receptors', *Biochemical and Biophysical Research Communications*, 201(1), pp. 449–457. doi: 10.1006/bbrc.1994.1722.

CHAPTER

3. Immunosuppressive role of Edn3 in the Melanoma Microenvironment

3.1. Introduction

The immunoediting concept suggests that the host immune system hampers tumor formation while shaping tumor immunogenicity. Immunoediting occurs in a stepwise process. During the elimination phase components of tumor immunosurveillance system encounter and demolish emerging tumors. Cancer cells that resist the forces of immunosurveillance are kept dormant during the equilibrium phase. Lastly, in the escape phase tumor cells that became less immunogenic form visible tumors. This last stage is empowered by the immunosuppressive setting established in the TME (Schreiber, Old and Smyth, 2011).

As demonstrated in Chapter 2, the melanoma microenvironment is characterized by infiltration of several immune cell populations. The intense recruitment of immune cells to melanoma tumors is a consequence of melanoma immunogenicity (Passarelli et al., 2017). Even though notably immunogenic, melanoma develops diverse mechanisms to evade immune detection. Indeed, melanoma is considered a highly immunosuppressive type of tumor (Umansky and Sevko, 2012). Inside the TME, suppressive mediators including cytokines and cells create an immunosuppressive network enabling tumors to escape from immune detection and destruction by engaging components of the immunosurveillance system (Liu, Workman and Vignali, 2016). Intratumoral regulatory T cells (Tregs) play a central role in contributing to the formation of the immunosuppressive milieu. Therefore, they support tumor development suppressing tumor-specific immune responses in the TME (Chaudhary and Elkord, 2016).

Tregs have strong immunosuppressive abilities that mediate self-tolerance, prevent autoimmune diseases and limit excessive immune responses. Tregs are a small population of CD4⁺ Helper T cells classically distinguished by the constitutive expression of CD25 and FOXP3 markers (Lee, 2017). CD25, the α -chain of IL-2 receptor, was used in the mid 90's to identify a small subset of mouse CD4⁺ Helper T cells responsible for maintaining self-tolerance (Sakaguchi *et al.*, 1995). Later it became clear that this population characterized as Tregs overexpresses CD25 and is able to suppress self-reactive T cells in mice (Suri-Payer *et al.*, 1998) as well as in humans (Baecher-Allan *et al.*, 2001; Ng *et al.*, 2001). Although CD25 is constitutively expressed by Tregs, non-suppressive cells such as CD8⁺ and CD4⁺ T cells transiently upregulate CD25 during T cell activation and inflammation (Chaudhary and Elkord, 2016; Liu, Workman and Vignali, 2016). Thus, identification of Tregs exclusively by CD25 is inaccurate and inclusion of other markers might be valuable (Fontenot *et al.*, 2005).

In the early 2000's the *forkhead box P3 (Foxp3)* gene was associated with the phenotype of scurfy mice. These mice develop fatal autoimmune disease due to spontaneous loss-of-function mutations in the *Foxp3* gene (Brunkow *et al.*, 2001). Similarly, humans with IPEX syndrome (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) harbor deleterious mutations in the *FOXP3* gene and develop strong autoimmune diseases (Bennett *et al.*, 2001). Shortly after the description of *FOXP3* gene, studies from Sakaguchi and Rudensky groups revealed that FOXP3 protein (also known as scurfin) is highly expressed by Tregs and helped to coin FOXP3 as a master regulator of Treg

function (Fontenot, Gavin and Rudensky, 2003; Hori, Nomura and Sakaguchi, 2003). FOXP3 regulates the expression of genes responsible for mediating Treg suppressive features (Zheng *et al.*, 2007; Frydrychowicz *et al.*, 2017).

Notwithstanding a variety of functions that sustain immune homeostasis, Tregs might also populate tumors promoting tolerance to tumor-associated antigens (TAAs) and suppressing anti-tumor immunity (Nishikawa and Sakaguchi, 2010). Tregs are routinely found in inflamed tumors that exhibit great amounts of CTLs and T_H1 cells (Togashi, Shitara and Nishikawa, 2019). Due to their highly immunosuppressive features and infiltration in the TME, Tregs are considered a critical hurdle to favorable outcome of immunotherapies (Chao and Savage, 2018). Intratumoral Tregs may disrupt the mechanisms of immunosurveillance and support tumor development (Yamaguchi and Sakaguchi, 2006; Takeuchi and Nishikawa, 2016; Togashi, Shitara and Nishikawa, 2019). Depletion of tumor resident Tregs results in increased infiltration of tumor inhibitory cells (Li *et al.*, 2010) accompanied by effective anti-tumor immune response (Shimizu *et al.*, 1999).

Tregs impair the immune response against tumors mediated by T_H1 CD4⁺ T Helper cells (Casares *et al.*, 2003), DCs (Chen *et al.*, 2017), CTLs (Chen *et al.*, 2005) and NK cells (Pedroza-Pacheco, Madrigal and Saudemont, 2013). Tregs have four basic mechanisms of immunosuppression including inhibitory cytokines, IL-2 consumption, cytolysis and modulation of DCs functions (Vignali, Collison and Workman, 2008). A diagrammatic representation of the immunosuppressive

mechanisms of Tregs that limit the anti-tumor immunity in the TME is shown in Figure 3.1.

Tregs disrupt immune response against tumor through secretion of immunosuppressive cytokines TGF- β and IL-10 (Yi et al., 2013). TGF- β impairs the development, proliferation, and function of anti-tumorigenic cells in the TME (Pickup, Novitskiy and Moses, 2013). Accordingly, TGF- β derived from Tregs mitigates anti-tumor immunity (Hilchey *et al.*, 2007). There is a positive correlation between high levels of TGF- β and reduced survival in patients with melanoma (Tang *et al.*, 2015). Similarly, IL-10 allows evasion of tumor cells from immune destruction in TME (Mannino et al., 2015) and high expression of IL-10 is associated with poor prognosis for melanoma patients (Dummer et al., 1995; Nemunaitis et al., 2001). A key mechanism of IL-10 immunosuppression is the impairment of antigen presentation (Sato et al., 2011). IL-10 secreted by Tregs hampers immune response against tumors in a contact-independent mechanism (Bergmann *et al.*, 2007). IL-35 is another cytokine required for maximal Treg suppression action (Vignali, Collison and Workman, 2008). Tregs are the main source of IL-35 in the TME and this cytokine limits the generation of tumor-specific CTLs as well as promotes intratumoral T cell exhaustion. Furthermore, Treg-specific depletion of IL-35 hampers tumor growth (Turnis *et al.*, 2016).

Although Tregs do not produce IL-2 (Papiernik *et al.*, 1998), due to IL-2R α (CD25) overexpression they constitutively uptake the IL-2 available in the microenvironment (Thornton and Shevach, 1998). Competition for IL-2

concurrently results in Tregs proliferation and inhibition of effector T cell growth since it depletes the IL-2 required for effector T cell propagation (de la Rosa *et al.*, 2004). Early in tumor development, when effector T cells are still producing IL-2, the upregulation of IL-2R α in Tregs reduces the availability of this cytokine limiting T cell proliferation (Liu, Workman and Vignali, 2016).

Secretion of granzyme and perforin leading to cytolysis of target cells is classically associated with activity of cytotoxic cells (i.e. CTLs and NK). After encountering its target, a cytotoxic cell releases granule containing perforin and granzymes. Perforin creates pores in the target cell allowing granzyme to access the cytosol and activate caspases (Lieberman, 2003). Unexpectedly, upon activation, Tregs were shown to express high levels of granzyme B (GZB) and exhibited cytotoxicity against different human cell lines (Grossman *et al.*, 2004). GZB and perforin produced by intratumoral Tregs suppress the anti-tumor response of NKs and CTLs. It is estimated that 5-30% of tumor resident Tregs express GZB (Cao *et al.*, 2007).

DCs functions are controlled by Tregs and this regulatory mechanism indirectly affects the function of effector T cells given that functional DCs are required for activation of T cells (Vignali, Collison and Workman, 2008). Tregs constitutively express the inhibitory receptor CTLA-4 (Read, Malmström and Powrie, 2000). CD28 exhibited on the surface of naïve T cells competes with CTLA-4 for B7 expressed by DCs. Notably, CTLA-4 has stronger affinity for B7. The interaction of CTLA-4 with B7 co-stimulatory molecule inactivates DCs and consequently

prevents the activation of effector T cells (Lee, 2017). Tregs employ CTLA-4 to hamper tumor immunity as CTLA-4-deficient Tregs exhibit reduced suppression which resulted in improved immune response against tumor (Wing *et al.*, 2008).

Tregs represent only 2-10% of CD4⁺ T cells in the peripheral blood of healthy patients. In contrast, it is estimated that Tregs account for 20-50% of CD4⁺ T cells present in the TME (Takeuchi and Nishikawa, 2016; Togashi, Shitara and Nishikawa, 2019). The prognostic value of Treg infiltrate for patients with cancer is still contentious and depends on tumor site (DeLeeuw *et al.*, 2012). In many types of cancers heavy Treg infiltration is connected with reduced survival of patients (Nishikawa and Sakaguchi, 2010; Chaudhary and Elkord, 2016). Still, the absolute number of intratumoral Tregs is not always correlated with patients' survival (Sato *et al.*, 2005). It has been suggested that increased ratio between Tregs and effector lymphocytes is a more reliable prognostic indicator (Chaudhary and Elkord, 2016) since it demonstrates that Tregs are efficiently suppressing anti-tumor immune responses (Takeuchi and Nishikawa, 2016). High incidence of intratumoral Tregs is associated with poor prognosis in patients with melanoma (Knol *et al.*, 2011), glioblastoma (Sayour *et al.*, 2015) and many carcinomas including lung (Tao *et al.*, 2012), pancreatic (Tang *et al.*, 2014) and ovarian cancer (Curiel *et al.*, 2004). On the other hand, in patients with colon (Frey *et al.*, 2010) and gastric tumors (Haas *et al.*, 2009), whose tumorigenesis are associated with long term inflammation, the immunosuppression associated with Tregs provides a shielding effect against tumor formation. Thus, a high number of intratumoral Tregs

in these tumor types are linked to improved prognosis (Chaudhary and Elkord, 2016; Frydrychowicz *et al.*, 2017).

Targeting of Tregs considerably improves tumor immunity and during the past few years immunotherapy strategies aiming to mitigate Tregs suppressive mechanisms have reached promising outcomes (Lee, 2017). Immunotherapy using monoclonal antibodies against CTLA-4 (ipilimumab) promotes activation of effector T cells (Maker, Attia and Rosenberg, 2005) resulting in improved outcome for patients with metastatic melanoma (Schadendorf *et al.*, 2015). The mechanism of action of ipilimumab is still not completely understood. It is assumed that ipilimumab prevents Tregs from obstructing effector T cells activation mediated by DCs (Liu, Workman and Vignali, 2016; Lee, 2017). It has also been suggested that ipilimumab selectively depletes Tregs through an antibody-dependent cellular cytotoxic mechanism (Simpson *et al.*, 2013).

Chronic inflammation and long-term immunosuppression are uncontrolled immune events that impact tissue microenvironment and endorse tumor formation and development (Quail and Joyce, 2013; Palucka and Coussens, 2016). Treg-mediated immunosuppression in the melanoma microenvironment is a relevant mechanism of escape from immune detection during the immunoediting process (Jacobs *et al.*, 2012). Endothelins are considered pro-inflammatory cytokines given that they stimulate inflammatory response (Nett *et al.*, 2006). Despite extensive studies that explored the immunoregulatory features of endothelins, how these cytokines negatively regulate immune response, particularly anti-tumor

immunity, is still poorly understood. Furthermore, the effect of endothelin on immunosuppressive cells such as Tregs has not been elucidated. The present study addressed the inhibitory effect of endothelin signaling during the immune response against melanoma.

3.2. Results

3.2.1. Endothelin is required for immune escape of YUMMER1.7 cells

Highly immunogenic YUMMER1.7 cells were intradermally injected in wild-type and *K5-Edn3* mice to evaluate whether activation of endothelin signaling in the melanoma microenvironment is required for immune escape. YUMMER1.7 cells were originated from YUMM1.7 cells after rounds of UV radiation followed by clonal selection. YUMMER1.7 cells have a higher mutation load compared to YUMM1.7 cells that elicited anti-tumor response. YUMMER1.7 cells exhibited tumor regression after injection in immunocompetent mice. YUMMER1.7 cells generated small tumors in wild-type mice 5 days after injections. These tumors began to shrink 8 days after injection and completely regressed 19 days post-injection (Figure 3.2). Initially, YUMMER1.7 tumors developed in *K5-Edn3* mice had similar growth rate to those in wild-type mice. However, after a short period of relapse, tumors escaped regression and resumed growth at day 12. Tumor growth was maintained even after the complete regression observed in wild-type tumors (Figure 3.2). This finding suggests that the presence of endothelin in melanoma microenvironment may promote evasion from immune destruction.

3.2.2. Endothelin increases the expression of Tregs-associated markers

Real-time quantitative PCR was carried out to investigate whether overexpression of *Edn3* in the melanoma microenvironment affects the expression of Treg-associated markers. qRT-PCR revealed that YUMM1.7-GFP melanoma tumors from *K5-Edn3* mice had increased expression of *Foxp3*, *Tgf- β* and *Il-10* genes when compared to tumors from control mice (Figure 3.3).

Next, I sought to confirm if the augmented expression of these genes was maintained at proteic level. The expression of FOXP3 protein in YUMM1.7-GFP tumors was examined using Western Blotting. The result supported the qRT-PCR data and showed that *K5-Edn3* tumors exhibited remarkably higher expression of FOXP3 when compared to wild-type tumors. FOXP3 expression in experimental tumors was two times higher ($p < 0.05$) than control tumors (Figure 3.4). TGF- β and IL-10 are two inhibitory cytokines that mediates the suppressive functions of Tregs. The expression of these immunosuppressive cytokines was evaluated using different approaches. The visual inspection of four sections per tumor from YUMM1.7-GFP tumors ($n=4/\text{group}$) did not detect TGF- β expression in wild-type tumors while TGF- β expression was observed in the microenvironment of *K5-Edn3* tumors (Figure 3.5). This result is in accordance with qRT-PCR data. Interestingly, TGF- β expression was mostly detected in extracellular space suggesting suppression by TGF- β occurs in a contact-independent manner. The production of IL-10 cytokine in the YUMM1.7-GFP tumors was quantified using IL-10 ELISPOT. In *K5-Edn3* tumors there was a significantly increased number ($p < 0.01$) of IL-10⁺ spots when compared with wild-type tumors (Figure 3.6). *K5-Edn3* tumors had on

average 193 IL-10⁺ spots while wild-type tumors had an average number of 11 IL-10⁺ spots. This result corroborated the observation that at the mRNA level IL-10 expression is higher in *K5-Edn3* tumors.

Quantification of GZB expression was performed by immunofluorescence in YUMM1.7-GFP tumors. Experimental tumors showed approximately four times more GZB expression ($p > 0.01$) when compared to control tumors (Figure 3.7). To confirm that GZB expression is restricted to Tregs, FOXP3 should have been co-stained with GZB. Unfortunately, FOXP3 antibody did not work properly for immunofluorescence staining. Therefore, due to technical limitations it was not possible to rule out that other cell types in melanoma TME also express GZB.

3.2.3. Ednrb⁺ Tregs suppress cytotoxic cells

To address whether the anti-tumor response in melanoma is counterbalanced by intratumoral Tregs expressing Ednrb, flow cytometric data was used to calculate the relative number of Ednrb⁺ Tregs to CTLs. This approach can demonstrate if Tregs are efficiently suppressing anti-tumor immune responses. *K5-Edn3* tumors exhibited an Ednrb⁺ Tregs/CTL ratio three times higher ($p < 0.05$) compared to wild-type tumors (Figure 3.8). This finding indicates that activation of endothelin signaling in Tregs prevents expansion of CTLs.

3.2.4. Immune checkpoint and Ednrb inhibitor decreased melanoma growth

To test whether the combination of immune checkpoint inhibitor with Ednrb blockage is a viable strategy to prevent melanoma growth, *K5-Edn3* mice bearing YUMM1.7-GFP tumors were treated with CTLA-4 blocking antibody alone or in

combination with Ednrb inhibitor (BQ-788) for three weeks. Mice in the control group received treatment of DMSO along with isotype control and developed large tumors by the end of the treatment (average volume of 1,829 mm³) (Figure 3.9A). Tumors treated with anti-CTLA-4 exhibited a growth rate similar to the control group until day 13 of treatment. After that day, anti-CTLA-4 treatment significantly prevented tumor growth ($p < 0.05$) (Figure 3.9A). Tumors treated with anti-CTLA-4 had an average volume of 432.03 mm³ when treatment was concluded. Treatment exclusively with BQ-788 and in combination with anti-CTLA-4 remarkably reduced melanoma growth ($p < 0.0001$) (Figure 3.9A). Indeed, some mice in these groups showed complete tumor regression. Tumors responded to BQ-788 earlier than anti-CTLA-4 although the volume of tumors from these groups were not statically different ($p > 0.05$) (Figure 3.9A). The response to BQ-788 was so accentuated that no difference ($p > 0.05$) was observed when tumors treated only with BQ-788 were compared to tumors treated with combination of BQ-788 and anti-CTLA-4 (Figure 3.9B). The average tumor volume observed in BQ-788 group at the end of treatment was 8.8 mm³ and the combination group had an average volume of 11.18 mm³. Altogether this data suggests that YUMM1.7-GFP tumors exposed to high levels of Edn3 are sensitive to immune checkpoint inhibitor as well as to Ednrb blockage, although the response to BQ-788 seems to be more noticeable.

3.2.5. Endothelin suppresses expression of inflammatory proteins in melanoma TME

In order to identify novel factors impacted by endothelin overexpression in the melanoma microenvironment I performed an unbiased cytokine screen.

YUMM1.7-GFP tumors from wild-type and *K5-Edn3* mice were harvested and changes in protein expression were analyzed by RayBiotech® Quantibody Mouse Cytokine Antibody Array. After normalization to internal controls, a group of 15 cytokines was identified as differentially expressed ($p < 0.05$) when wild-type and *K5-Edn3* were compared (Figure 3.10A). *K5-Edn3* tumors exhibited decreased levels of IL-28 ($p < 0.05$) as well as the classical pro-inflammatory cytokine IL-17E ($p < 0.01$) (Figure 3.10B). These results are in accordance with my previous findings and confirmed that endothelin signaling promotes immunosuppression in the melanoma microenvironment.

3.3. Discussion

The present study suggests that endothelin is an important component of the immunoediting process that takes place in melanoma development, particularly during the escape phase. YUMMER1.7 melanoma cells, which harbor a high mutational load, developed tumors in immunocompetent mice. These tumors were possibly detected by the tumor immunosurveillance system eliciting anti-tumor responses and tumor regression. A recent study demonstrated that YUMMER1.7 tumor regression in immunocompetent mice is a process that relies on cells from the adaptive immune response (Wang *et al.*, 2017). Tumors growing without abundant endothelin in the microenvironment regressed completely around 20 days after injection. Conversely, when melanoma tumors grew in an End3-rich microenvironment they overcame immune destruction and evolved to tumors that did not regress. Therefore, the presence of endothelin in melanoma microenvironment facilitates the evasion from anti-tumor immunity.

Tregs suppress immune response against tumors and are considered central mediators for the escape from immune destruction (Takeuchi and Nishikawa, 2016). Tregs infiltration is routinely found in patients with melanoma and these cells are considered functionally immunosuppressive (Vence *et al.*, 2007). This study supports the notion that Tregs orchestrate immunosuppression in melanoma microenvironment. Enrichment of Tregs in melanoma tumors is generally associated with poor survival (Miracco *et al.*, 2007; Mougiakakos *et al.*, 2010; Gerber *et al.*, 2014), although some studies did not find a correlation between Treg infiltration and melanoma outcome (Hillen *et al.*, 2008; Ladányi *et al.*, 2010). This discrepancy is probably due to the use of absolute number of Tregs rather than relative amount of Tregs to effector lymphocytes. Several preclinical studies have shown that a better outcome is achieved when melanoma tumors exhibited a ratio Treg/effector lymphocyte that favors effector cells (Nair *et al.*, 2007; Cohen *et al.*, 2010; Shabaneh *et al.*, 2018). Accordingly, less aggressive YUMM1.7-GFP tumors had a smaller ratio of Ednrb⁺ Tregs/CTLs when compared to aggressive tumors. This finding suggests that more CTLs are present in melanomas when there is no activation of endothelin signaling in Tregs. Thus, tumor immunity in melanoma microenvironment is hampered when Tregs are exposed to high levels of Edn3.

Tregs are defined by the constitutive expression of CD25 and FOXP3 markers (Lee, 2017). Yet, CD25 is transiently expressed by CD8⁺ and CD4⁺ T cells (Liu, Workman and Vignali, 2016). It is thus possible that cells other than Tregs might have contributed to the increased numbers CD25⁺ cells observed in tumors growing in *K5-Edn3* mice. FOXP3 is regarded as a reliable marker for murine

Tregs (Vignali, Collison and Workman, 2008). *K5-Edn3* tumors showed augmented FOXP3 expression at mRNA and proteic levels relative to wild-type tumors confirming that there is an enrichment of Tregs when End3 is overexpressed in melanoma microenvironment. The mechanism adopted by endothelin to govern the accumulation of Tregs in *K5-Edn3* tumors remains unclear. It is possible that endothelin impacted Tregs recruitment, proliferation, or conversion of conventional T cells into Tregs (Lee, 2017). The main mechanism responsible for Treg accumulation in tumors relies on chemokines system (Wang, Lee and Kim, 2012; Plitas *et al.*, 2016). Given that the chemical structure of mature endothelin is very similar to that of other chemokines (Grimshaw, Wilson and Balkwill, 2002) it is tempting to claim that endothelin regulates the chemotactic trafficking of Tregs to melanoma microenvironment.

Tumor intrinsic factors such as driver mutations might tolerate antitumor immunity in addition to promoting tumor formation (Togashi, Shitara and Nishikawa, 2019). A recent study demonstrated that early during tumorigenesis BRAF^{V600E} is responsible for recruitment of Tregs to melanoma microenvironment and impaired tumor surveillance (Shabaneh *et al.*, 2018). Importantly, Tregs are required for immune escape of BRAF^{V600E} mutated YUMMER1.7 melanoma cells (Wang *et al.*, 2017). The current study validates these previous findings. YUMM1.7-GFP melanoma cells, which harbor BRAF^{V600E} mutation, developed tumors that express Treg marker FOXP3 in both wild-type and *K5-Edn3* mice. Nevertheless, in *K5-Edn3* tumors FOXP3 expression was augmented. This suggests that in an endothelin-rich microenvironment Tregs suppressive functions

are amplified. FOXP3 is considered the master regulator of Treg function (Togashi, Shitara and Nishikawa, 2019). Indeed, this transcription factor regulates the expression of genes that mediate the immunosuppressive features of Tregs including surface proteins CD25 and CTLA-4 and effector molecules IL-10 and GZB (Zheng *et al.*, 2007). Genome-wide methylation comparison between human Treg and naïve T cell revealed 127 regions differentially methylated (RDM). Interestingly, EDNRB gene is among these RDMs in Tregs. EDNRB gene is hypomethylated and is considered a putative binding site of FOXP3. This finding implicates that EDNRB expression in Tregs is under the control of transcription factor FOXP3 (Zhang *et al.*, 2013).

Levels of immunosuppressive cytokines TGF- β and IL-10 were elevated in *K5-Edn3* tumors at both mRNA and proteic level indicating that endothelin stimulates the secretion of immunosuppressive cytokines in melanoma microenvironment. Since *K5-Edn3* tumors exhibited enrichment of Tregs it is possible that these suppressive cells accounted for the augmented level of TGF- β and IL-10 in an endothelin-rich microenvironment. There is limited information regarding the association between endothelins and immunosuppressive cytokines IL-10 and TGF- β . It has been demonstrated that in mouse aorta and mesenteric artery IL-10 antagonizes the vascular responses to Edn1 (Giachini *et al.*, 2009). Consequently, in the context of vascular stimulation endothelin and IL-10 have conflicting effects. This is in contrast with the observed functions of endothelin and IL-10 in *K5-Edn3* tumors. Consistent with the present study, macrophages treated with endothelin exhibited increased expression of IL-10 and TGF- β (Soldano *et al.*, 2016).

Furthermore, TGF- β and Edn1 displayed synergistic effects during vascular remodeling (Lambers *et al.*, 2013).

Tregs also employ contact-dependent mechanism to suppress effector cells, including production of GZB. It has already been demonstrated that production of GZB by intratumoral Tregs suppresses the anti-tumor response of NKs and CTLs (Cao *et al.*, 2007). Single-cell RNA sequencing showed that Tregs isolated from melanoma tumors express GZB (Tirosh *et al.*, 2016). GZB expression was accentuated in *K5-Edn3* tumors. Given the increased ratio Ednrb⁺ Tregs/CTLs in *K5-Edn3* tumors it is possible that the activation of endothelin signaling in Tregs is responsible for Treg-mediated cytotoxicity of CTLs. However, it is important to note that other cell types such as CTLs and NKs also express GZB (Lieberman, 2003). The data presented in this study is not sufficient to claim that in YUMM1.7-GFP tumors GZB expression is restricted to Tregs. Given that *K5-Edn3* tumors exhibited a higher number of Tregs it is reasonable to propose that Tregs influenced the augmented GZB expression in *K5-Edn3* tumors.

Altogether these data suggest that when YUMM1.7-GFP tumors are exposed to a microenvironment rich in Edn3, the expression of markers associated with Treg function is augmented. Interestingly, markers of both contact-independent and contact-dependent mechanisms of Treg suppression were enhanced in *K5-Edn3* tumors. These findings implicate that endothelin signaling is required for immunosuppressive features of Tregs. Unexpectedly, endothelin which is

generally considered a pro-inflammatory cytokine seems to play an immunosuppressive role in the microenvironment of YUMM1.7-GFP melanoma.

Therapeutic approaches aiming to block Treg functions have produced clinical success in melanoma. Immunotherapy using monoclonal antibodies against CTLA-4 (ipilimumab) results in improved outcome for patients with metastatic melanoma (Schadendorf *et al.*, 2015). Blockage of Ednrb efficiently reduced melanoma growth *in vitro* and *in vivo* (Lahav, Heffner and Patterson, 1999). The present study investigated for the first time if the combination of Ednrb inhibitor (BQ-788) with immunotherapy using CTLA-4 blocking antibody is a therapeutic strategy that abrogate melanoma growth more effectively. YUMM1.7-GFP tumors exposed to a microenvironment rich in Edn3 responded to immune checkpoint inhibitor. YUMM1.7-GFP tumors exhibited a delayed response to anti-CTLA-4, which has been previously reported in patients with melanoma (Saenger and Wolchok, 2008). The mechanisms of action of CTLA-4 blocking antibody was not explored in detail in the *K5-Edn3* mouse model. However, based on the elevated number of Tregs when YUMM1.7-GFP tumors are exposed to high levels of Edn3 is possible to argue that anti-CTLA-4 prevented the T cell activation mediated by Tregs or even promoted Treg depletion (Togashi, Shitara and Nishikawa, 2019).

Several *in vitro* and preclinical studies have demonstrated the anti-cancer effect of Ednrb blockage in melanoma (Lahav, Heffner and Patterson, 1999; Rosanò *et al.*, 2004; Smith *et al.*, 2017). The anti-tumor effect of Ednrb inhibition was more pronounced than immune checkpoint inhibitor in YUMM1.7-GFP tumors. Along

these lines, the potent effect of BQ-788 against melanoma has already been shown to be stronger than other therapeutic approaches such as BRAF inhibition (Smith *et al.*, 2017). Combination of anti-CTLA-4 with BQ-788 produced the same anti-tumoral effect as BQ-788 alone, suggesting that Ednrb blockage is sufficient to prevent melanoma growth. The anti-tumor activity of anti-CTLA-4 was probably restricted to Tregs. Conversely, the striking anti-melanoma effect of BQ-788 might have been the consequence of Ednrb signaling blockage in Tregs as well as other cells in the TME. A recent study demonstrated that intratumoral administration of BQ-788 in melanoma patients reduced tumor growth and enhanced immune cell infiltration (Wouters *et al.*, 2015). These data along with the present study support the idea that Ednrb blockage might effectively be employed as a therapeutic strategy for melanoma patients that express high levels of components of endothelin signaling.

The present study indicates that endothelin presence in melanoma microenvironment enables the formation of an immunosuppressive milieu facilitating escape from tumor immunity. Tumors growing in abundance of endothelin exhibited remarkable reduction in the expression of chemokines and pro-inflammatory cytokines. Surprisingly, in *K5-Edn3* tumors there was a significant reduction in the expression of chemokines CXCL11, CXCL2, CXCL12, CCL12 and CCL9. Chemokines regulate the recruitment of different populations of immune cells to TME (Nagarsheth, Wicha and Zou, 2017). Since immune cell populations (other than Tregs) did not differ when wild-type and *K5-Edn3* tumors

were compared, it is possible that the presence of endothelin did not influence the overall trafficking of immune cells to melanoma microenvironment.

IL-17 is a pro-inflammatory cytokine required for anti-tumor features of T_H17 cells during tumor immunosurveillance (Grivennikov, Greten and Karin, 2010). T_H17 is an effector cell of tumor immunity and its presence in TME is associated with diminished numbers of Treg and increased amounts of CTLs and NK cells (Zou and Restifo, 2010). Reduction in the levels of IL-17F in *K5-Edn3* tumors indicated that endothelin impaired tumor immunity in the YUMM1.7-GFP melanoma tumors. It has been shown that activation of EdnrA, but not EdnrB, stimulates the production of IL-17 in T_H17 cells (Tanaka *et al.*, 2014). Based on the observation that in presence of Edn3 there is downregulation of IL-17 it is plausible to argue that endothelin receptors might play ambiguous roles in T_H17 cells as they do in DCs (Guruli *et al.*, 2004). IL-28 is another cytokine that elicits anti-tumor response (Numasaki *et al.*, 2007). Downregulation of IL-28 cytokine in *K5-Edn3* tumors was of particular interest given that the association between this cytokine and endothelin has not been previously described. The reduced levels of IL-28 in *K5-Edn3* tumors corroborated the observation that endothelin weakened tumor immunity.

During the last few years, a growing body of data have challenged the paradigm that endothelin exclusively stimulates inflammatory response. Expression of EdnrB on DCs decrease their maturation and survival suggesting that EdnrB negatively regulates immune response (Guruli *et al.*, 2004). Endothelin promotes

macrophage polarization towards M2 phenotype, which are distinguished by their anti-inflammatory features. Indeed, Edn1 treatment stimulates macrophages to secrete immunosuppressive cytokines IL-10 and TGF- β (Soldano *et al.*, 2016). EDNRB expression on endothelial cells impairs the homing of anti-tumor immune cells to TME (Aubert and Juillerat-Jeanneret, 2016). EDNRB decreases the expression of adhesion molecule ICAM-1 resulting in reduced recruitment of CTLs to ovarian cancer microenvironment. The negative impact of endothelin on lymphocyte homing is sustained even in presence of pro-inflammatory cytokine TNF- α . Hence, EDNRB activation in intratumoral endothelial cells creates an endothelial barrier that nourishes immune-privileged microenvironment. Importantly, EDNRB blockage increased the expression of adhesion molecules ICAM-1 in the vasculature followed by augmented homing of CTLs to TME (Buckanovich *et al.*, 2008). Overexpression of EDNRB in endothelial cells from aggressive glioblastomas is associated with less CTL infiltrate and more Tregs present in TME. This finding implicates that high expression of EDNRB in TME impairs recruitment of tumor inhibitory immune cells while contributes to evasion of immune detection (Nakashima *et al.*, 2016). These data together with the present study advocates for an immunosuppressive role of endothelin in TME resulting in escape from tumor immunity.

3.4. Methods

3.4.1. Tumor cell injection

A total of 1,000,000 YUMMER1.7 melanoma cells (Wang et al., 2017) were intradermally injected in the shaved flank of *K5-Edn3* or wild-type mice as described in 2.4.3. YUMMER1.7 cells were a gift from Dr. Marcus Bosenberg (Yale University). The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Florida International University protocol number 16-018.

3.4.2. *In vivo* experiments

K5-Edn3 mice were intradermally injected with 100,000 YUMM1.7-GFP as described in 2.4.3. When tumors became palpable (7 days after injection) mice were randomized into 4 groups (n=3) to receive the treatments three times per week for three weeks. Mice in the control group received intraperitoneal injection of DMSO and isotype control Syrian Hamster IgG2 (Bio X Cell, BP0087). Mice in the *Ednrb* inhibitor group were intraperitoneally treated with 10 mg/kg of BQ-788 (ApexBio, BP3278). Mice in the immunotherapy group were treated with 10 mg/kg of anti-CTLA-4 (Bio X Cell, BP0131) clone 9H10 via intraperitoneal. Mice in the combination group were simultaneous treated with BQ-788 and anti-CTLA-4. Tumor volume was calculated as described in 2.4.3 and statistical analysis was carried out with One-Way ANOVA and considered to be significantly different at $p < 0.05$.

3.4.3. Real-time Quantitative PCR (qRT-PCR)

Total RNA from YUMM1.7-GFP tumors was extracted using Direct-Zol® RNA MiniPrep Plus Kit (Zymo Research, R2070). Complementary DNA (cDNA) synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622). Real-time quantitative PCR (qRT-PCR) was conducted using Thermo Scientific Maxima SYBR Green Master Mix (Thermo Scientific, K0221). Each sample was analyzed in triplicates and the relative mRNA expression levels of *FOXP3*, *TGF-β* and *IL-10* were normalized to those of *GAPDH*. Primers sequences were as follows: *FOXP3*: forward CCCAGGAAAGACAGCAACCTT, reverse TTCTCACAACCAGGCCACTTG; *TGF-β*: forward CCTGCAAGACCATCGACATG, reverse TGTTGTACAAAGCGAGCACC; *IL-10*: forward ATAACTGCACCCACTTCCCA, reverse GGGCATCACTTCTACCAGGT; *GAPDH*: forward TGCAGTGGCAAAGTGGAGAT, reverse TTTGCCGTGAGTGGAGTCATA.

3.4.4. Western Blotting

Harvested YUMM1.7-GFP tumors were lysed using RIPA lysis buffer. Tissue lysates were centrifuged at 16,000rpm at 4°C for 15min. The supernatant was collected and denaturated using 4x NuPAGE LDS Sample Buffer with 5% β-mercaptoethanol. Pre-cast 4-12% SDS-PAGE gels were used to analyze the protein samples which were subsequently transferred to PVDF membrane. The membranes were incubated with *FOXP3* (1:1,000, Proteintech, 22228-1-AP) and β-actin (1:4,000, Proteintech, 20536-1-AP) antibodies followed by HRP conjugated secondary antibody (1:5,000, Cell Signaling Technology, 7074) incubation. Finally,

the labeled proteins were visualized using chemiluminescence kit (Thermo Scientific, 34577). Bands intensity were quantified using ImageJ software. Statistical analysis was carried out with Student T-Test and considered to be significantly different at $p < 0.05$.

3.4.5. Immunofluorescence Analysis

TGF- β (1:100, Abcam, ab92486) and GZB (1:100, Abcam, ab53097) primary antibodies were used for immunofluorescence staining. The procedure was performed as described in 2.4.5. GZB immunofluorescence staining was quantified in terms of mean fluorescence intensity using ImageJ software. Twenty random fields of four sections per tumor ($n=4$ /group) were analyzed. Statistical analysis was carried out with Student T-Test and considered to be significantly different at $p < 0.05$.

3.4.6. IL-10 ELISPOT

MultiScreen® 96-well plates (Millipore, MAIPS4510) were coated with mouse IL-10 ELISPOT detection antibody (BD Biosciences, 51-9002752) and incubated overnight at 4°C, washed three times with PBS and blocked with DMEM 10% FBS for 2h at 37°C. Single-cells suspension from harvest YUMM1.7-GFP tumors were prepared as described in 2.4.4. A total of 1,000,000 cells derived from tumor dissociation were seeded in triplicates and incubated for 24h. After incubation wells were washed three times with wash buffer (0.05% Tween-20 in PBS) and incubated for 2h at room temperature with biotinylated mouse IL-10 ELISPOT capture antibody (BD Biosciences, 51-9002772). Following incubation, wells were

washed three times with wash buffer and incubated with avidin-HPR (Invitrogen, 18-4100-51) for 2h at room temperature. Spots were developed after incubation with AEC Substrate Solution for 3min at room temperature. IL-10⁺ spots were counted using CTL Immunospot S6 ELISPOT reader. Statistical analysis was carried out with Student T-Test and considered to be significantly different at $p < 0.05$.

3.4.7. Cytokine array

Protein homogenates were prepared as described in 3.4.4. RayBiotech[®] Quantibody Mouse Cytokine Antibody Array (QAM-CAA-4000; RayBiotech) was conducted by RayBiotech. This assay simultaneously detects and quantifies 200 cytokines and consisted of a multiplexed sandwich ELISA-based quantitative array platform. The fluorescence intensity levels were normalized to internal positive controls present in each array. Statistical analysis was carried out with Student T-Test and proteins were considered differentially expressed when $p < 0.05$.

3.5. Figures

Figure 3.1 Immunosuppressive mechanisms of Treg in TME

Tregs have many immunosuppressive mechanisms that limit anti-tumor immunity in TME. Tregs produce immunosuppressive cytokines TGF- β , IL-10 and IL-35 and uptake available IL-2 through overexpression of CD25. Tregs also express granzyme B (GZB) to directly kill CTLs and NK cells and prevent the co-stimulatory signaling in DCs through expression of immune checkpoint CTLA-4.

YUMMER1.7 intradermal

Figure 3.2 Endothelin is required for immune escape of YUMMER1.7 melanoma cells

Tumor volume of mice (n=5) that received intradermal injections of YUMMER1.7 melanoma cells. ** $p < 0.01$

Figure 3.3 Endothelin increases the expression of Tregs-associated markers in the YUMM1.7-GFP melanoma tumors

qRT-PCR analysis of *Foxp3*, *Tgf- β* and *Il-10* mRNA levels in wild-type and *K5-Edn3* tumors. Fold change was calculated as the relative mRNA amount of target gene in wild-type and *K5-Edn3* tumors normalized to *GAPDH*. Shown is one of the three experiments performed.

A **B**

Figure 3.4 Endothelin increases FOXP3 expression in YUMM1.7-GFP melanoma tumors

A. Western blot analysis of FOXP3 and β-actin expression in intradermally injected YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice. **B.** Densitometric analysis of FOXP3 expression relative to β-actin expression (n=3/group). * $p<0.05$.

Figure 3.5 Endothelin increases TGF- β expression in YUMM1.7-GFP melanoma tumors

Immunofluorescence staining of intradermally injected YUMM1.7-GFP tumors of wild-type (**A**) and *K5-Edn3* (**B**) mice for TGF- β . TGF- β expression was observed only in *K5-Edn3* tumors.

Figure 3.6 Endothelin increases IL-10 expression in YUMM1.7-GFP melanoma tumors

A. Representative wells of IL-10 ELISPOT of intradermal YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice. **B.** Quantification of IL-10⁺ cells. ** $p < 0.01$. Shown is one of the two experiments performed.

Figure 3.7 Endothelin increases granzyme B (GZB) expression in YUMM1.7-GFP melanoma tumors

A. Immunofluorescence staining in YUMM1.7-GFP intradermal tumors of wild-type (left) and *K5-Edn3* (center) mice for Granzyme. **B.** Mean fluorescence intensity analysis of 20 random fields per tumor (n=4/group). ** $p < 0.01$.

Figure 3.8 Endothelin increases the number of Ednrb⁺ Tregs relative to cytotoxic cells in YUMM1.7-GFP melanoma tumors

Flow cytometric quantification of Treg Ednrb⁺ relative to CTLs cells from intradermal YUMM1.7-GFP tumors of wild-type and *K5-Edn3* mice (n=3/group). **p*<0.05.

A

B

Figure 3.9 Immune checkpoint and Ednrb inhibitor decreased melanoma growth

K5-Edn3 mice bearing YUMM1.7-GFP tumors were treated with BQ-788 (10 mg/Kg) alone, anti-CTLA-4 (10 mg/Kg) alone or in combination three times a week for three weeks. **A.** Tumor volume of *K5-Edn3* mice that received drug treatments (n=3). **B.** Representative tumor of each group after resection. * $p < 0.05$, *** $p < 0.0001$

Figure 3.10 Endothelin suppress the expression of inflammatory proteins in YUMM1.7-GFP melanoma tumors

Changes in protein expression of the YUMM1.7-GFP tumors of wild-type and *K5-Edn3* were analyzed by RayBiotech Quantibody® Mouse Cytokine Array. The fluorescence intensity levels were normalized to internal positive controls present in each array. Normalized fluorescence intensity for 15 differentially expressed proteins ($p < 0.05$) are represented in the heat map (A) and as the mean $\pm$ S.E.M. (B) ($n = 3/\text{group}$). * $p < 0.05$, ** $p < 0.01$.

3.6. References

- Aubert, J. D. and Juillerat-Jeanneret, L. (2016) 'Endothelin-Receptor Antagonists beyond Pulmonary Arterial Hypertension: Cancer and Fibrosis', *Journal of Medicinal Chemistry*, 59(18), pp. 8168–8188. doi: 10.1021/acs.jmedchem.5b01781.
- Baecher-Allan, C. *et al.* (2001) 'CD4+CD25^{high} Regulatory Cells in Human Peripheral Blood', *The Journal of Immunology*, 167(3), pp. 1245–1253. doi: 10.4049/jimmunol.167.3.1245.
- Bennett, C. L. *et al.* (2001) 'The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3', *Nature Genetics*, 27(1), pp. 20–21. doi: 10.1038/83713.
- Bergmann, C. *et al.* (2007) 'Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment', *Cancer Immunology, Immunotherapy*, 56(9), pp. 1429–1442. doi: 10.1007/s00262-007-0280-9.
- Brunkow, M. E. *et al.* (2001) 'Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse', *Nature Genetics*, 27(1), pp. 68–73. doi: 10.1038/83784.
- Buckanovich, R. J. *et al.* (2008) 'Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy.', *Nature medicine*, 14(1), pp. 28–36. doi: 10.1038/nm1699.
- Cao, X. *et al.* (2007) 'Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance', *Immunity*, 27(4), pp. 635–646. doi: 10.1016/j.immuni.2007.08.014.
- Casares, N. *et al.* (2003) 'CD4⁺/CD25⁺ Regulatory Cells Inhibit Activation of Tumor-Primed CD4⁺ T Cells with IFN- γ -Dependent Antiangiogenic Activity, as well as Long-Lasting Tumor Immunity Elicited by Peptide Vaccination', *The Journal of Immunology*, 171(11), pp. 5931–5939. doi: 10.4049/jimmunol.171.11.5931.
- Chao, J. L. and Savage, P. A. (2018) 'Unlocking the Complexities of Tumor-Associated Regulatory T Cells', *The Journal of Immunology*, 200(2), pp. 415–421. doi: 10.4049/jimmunol.1701188.
- Chaudhary, B. and Elkord, E. (2016) 'Regulatory T Cells in the Tumor Microenvironment and Cancer Progression: Role and Therapeutic Targeting', *Vaccines*, 4(3), p. 28. doi: 10.3390/vaccines4030028.
- Chen, M.-L. *et al.* (2005) 'Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF- β signals in vivo', *Proceedings of the National Academy of Sciences*, 102(2), pp. 419–424. doi: 10.1073/pnas.0408197102.

- Chen, X. *et al.* (2017) 'Tumor-derived CD4+CD25+regulatory T cells inhibit dendritic cells function by CTLA-4', *Pathology - Research and Practice*. Elsevier GmbH., 213(3), pp. 245–249. doi: 10.1016/j.prp.2016.12.008.
- Cohen, A. D. *et al.* (2010) 'Agonist Anti-GITR Monoclonal Antibody Induces Melanoma Tumor Immunity in Mice by Altering Regulatory T Cell Stability and Intra-Tumor Accumulation', *PLoS ONE*. Edited by D. Unutmaz, 5(5), p. e10436. doi: 10.1371/journal.pone.0010436.
- Curiel, T. J. *et al.* (2004) 'Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival', *Nature Medicine*, 10(9), pp. 942–949. doi: 10.1038/nm1093.
- DeLeeuw, R. J. *et al.* (2012) 'The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: A critical review of the literature', *Clinical Cancer Research*, 18(11), pp. 3022–3029. doi: 10.1158/1078-0432.CCR-11-3216.
- Dummer, W. *et al.* (1995) 'Elevated serum levels of interleukin-10 in patients with metastatic malignant melanoma', *Melanoma Res*, 5(1), pp. 67–68.
- Fontenot, J. D. *et al.* (2005) 'Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3', *Immunity*, 22(3), pp. 329–341. doi: 10.1016/j.immuni.2005.01.016.
- Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003) 'Foxp3 programs the development and function of CD4+CD25+ regulatory T cells', *Nature Immunology*, 4(4), pp. 330–336. doi: 10.1038/ni904.
- Frey, D. M. *et al.* (2010) 'High frequency of tumor-infiltrating FOXP3+regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients', *International Journal of Cancer*, 126(11), pp. 2635–2643. doi: 10.1002/ijc.24989.
- Frydrychowicz, M. *et al.* (2017) 'The Dual Role of Treg in Cancer', *Scandinavian Journal of Immunology*, 86(6), pp. 436–443. doi: 10.1111/sji.12615.
- Gerber, A. L. *et al.* (2014) 'High expression of FOXP3 in primary melanoma is associated with tumour progression', *British Journal of Dermatology*, 170(1), pp. 103–109. doi: 10.1111/bjd.12641.
- Giachini, F. R. C. *et al.* (2009) 'Interleukin-10 attenuates vascular responses to endothelin-1 via effects on ERK1/2-dependent pathway', *American Journal of Physiology-Heart and Circulatory Physiology*, 296(2), pp. H489–H496. doi: 10.1152/ajpheart.00251.2008.
- Grimshaw, M. J., Wilson, J. L. and Balkwill, F. R. (2002) 'Endothelin-2 is a macrophage chemoattractant: Implications for macrophage distribution in tumors', *European Journal of Immunology*, 32(9), pp. 2393–2400. doi: 10.1002/1521-4141(200209)32:9<2393::AID-IMMU2393>3.0.CO;2-4.

- Grivennikov, S. I., Greten, F. R. and Karin, M. (2010) 'Immunity, Inflammation, and Cancer', *Cell*. Elsevier Inc., 140(6), pp. 883–899. doi: 10.1016/j.cell.2010.01.025.
- Grossman, W. *et al.* (2004) 'Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells', *Blood*, 104(9), pp. 2840–2848. doi: 10.1182/blood-2004-03-0859.
- Guruli, G. *et al.* (2004) 'Function and survival of dendritic cells depend on endothelin-1 and endothelin receptor autocrine loops', *Blood*, 104(7), pp. 2107–2115. doi: 10.1182/blood-2003-10-3559.
- Haas, M. *et al.* (2009) 'Stromal regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia', *BMC Gastroenterology*, 9(1), p. 65. doi: 10.1186/1471-230X-9-65.
- Hilchey, S. P. *et al.* (2007) 'Follicular Lymphoma Intratumoral CD4+CD25+GITR+ Regulatory T Cells Potently Suppress CD3/CD28-Costimulated Autologous and Allogeneic CD8+CD25- and CD4+CD25- T Cells', *The Journal of Immunology*, 178(7), pp. 4051–4061. doi: 10.4049/jimmunol.178.7.4051.
- Hillen, F. *et al.* (2008) 'Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma', *Cancer Immunology, Immunotherapy*, 57(1), pp. 97–106. doi: 10.1007/s00262-007-0353-9.
- Hori, S., Nomura, T. and Sakaguchi, S. (2003) 'Control of Regulatory T Cell Development by the Transcription Factor Foxp3', *Science*, 299(5609), pp. 1057–1061. doi: 10.1126/science.1079490.
- Jacobs, J. F. M. *et al.* (2012) 'Regulatory T cells in melanoma: The final hurdle towards effective immunotherapy?', *The Lancet Oncology*, 13(1), pp. 32–42. doi: 10.1016/S1470-2045(11)70155-3.
- Knol, A. C. *et al.* (2011) 'Prognostic value of tumor-infiltrating Foxp3+ T-cell subpopulations in metastatic melanoma', *Experimental Dermatology*, 20(5), pp. 430–434. doi: 10.1111/j.1600-0625.2011.01260.x.
- de la Rosa, M. *et al.* (2004) 'Interleukin-2 is essential for CD4+CD25+ regulatory T cell function', *European Journal of Immunology*, 34(9), pp. 2480–2488. doi: 10.1002/eji.200425274.
- Ladányi, A. *et al.* (2010) 'FOXP3+ Cell Density in Primary Tumor Has No Prognostic Impact in Patients with Cutaneous Malignant Melanoma', *Pathology & Oncology Research*, 16(3), pp. 303–309. doi: 10.1007/s12253-010-9254-x.
- Lahav, R., Heffner, G. and Patterson, P. H. (1999) 'An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(20), pp. 11496–11500.

- Lambers, C. *et al.* (2013) 'The Interaction of Endothelin-1 and TGF- β 1 Mediates Vascular Cell Remodeling', *PLoS ONE*. Edited by N. Frangogiannis, 8(8), p. e73399. doi: 10.1371/journal.pone.0073399.
- Lee, G. R. (2017) 'Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells', *Mediators of inflammation*, 2017, pp. 1–9. doi: 10.1155/2017/5458178.
- Li, X. *et al.* (2010) 'Efficient Treg depletion induces T-cell infiltration and rejection of large tumors', *European Journal of Immunology*, 40(12), pp. 3325–3335. doi: 10.1002/eji.201041093.
- Lieberman, J. (2003) 'The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal', *Nature Reviews Immunology*, 3(5), pp. 361–370. doi: 10.1038/nri1083.
- Liu, C., Workman, C. J. and Vignali, D. A. A. (2016) 'Targeting regulatory T cells in tumors', *FEBS Journal*, 283, pp. 2731–2748. doi: 10.1111/febs.13656.
- Maker, A. V., Attia, P. and Rosenberg, S. A. (2005) 'Analysis of the Cellular Mechanism of Antitumor Responses and Autoimmunity in Patients Treated with CTLA-4 Blockade', *The Journal of Immunology*, 175(11), pp. 7746–7754. doi: 10.4049/jimmunol.175.11.7746.
- Mannino, M. H. *et al.* (2015) 'The paradoxical role of IL-10 in immunity and cancer', *Cancer Letters*. Elsevier Ireland Ltd, 367(2), pp. 103–107. doi: 10.1016/j.canlet.2015.07.009.
- Miracco, C. *et al.* (2007) 'Utility of tumour-infiltrating CD25+FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma', *Oncology Reports*, 18(5), pp. 1115–1122.
- Mougiakakos, D. *et al.* (2010) 'Intratumoral forkhead box p3-positive regulatory t cells predict poor survival in cyclooxygenase-2-positive uveal melanoma', *Cancer*, 116(9), pp. 2224–2233. doi: 10.1002/cncr.24999.
- Nagarsheth, N., Wicha, M. S. and Zou, W. (2017) 'Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy', *Nature Reviews Immunology*. Nature Publishing Group, 17(9), pp. 559–572. doi: 10.1038/nri.2017.49.
- Nair, S. *et al.* (2007) 'Vaccination against the Forkhead Family Transcription Factor Foxp3 Enhances Tumor Immunity', *Cancer Research*, 67(1), pp. 371–380. doi: 10.1158/0008-5472.CAN-06-2903.
- Nakashima, S. *et al.* (2016) 'Endothelin B receptor expression in malignant gliomas: the perivascular immune escape mechanism of gliomas', *Journal of Neuro-Oncology*. Springer US, 127(1), pp. 23–32. doi: 10.1007/s11060-015-2017-5.

- Nemunaitis, J. *et al.* (2001) 'Comparison of Serum Interleukin-10 (IL-10) Levels Between Normal Volunteers and Patients with Advanced Melanoma', *Cancer Investigation*, 19(3), pp. 239–247. doi: 10.1081/CNV-100102550.
- Nett, P. C. *et al.* (2006) 'Recent developments on endothelin antagonists as immunomodulatory drugs--from infection to transplantation medicine.', *Recent patents on cardiovascular drug discovery*, 1(3), pp. 265–276. doi: 10.2174/157489006778776990.
- Ng, W. F. *et al.* (2001) 'Human CD4 + CD25 + cells: a naturally occurring population of regulatory T cells', *Blood*, 98(9), pp. 2736–2744. doi: 10.1182/blood.V98.9.2736.
- Nishikawa, H. and Sakaguchi, S. (2010) 'Regulatory T cells in tumor immunity', *International Journal of Cancer*, 127(4), pp. 759–767. doi: 10.1002/ijc.25429.
- Numasaki, M. *et al.* (2007) 'IL-28 Elicits Antitumor Responses against Murine Fibrosarcoma', *The Journal of Immunology*, 178(8), pp. 5086–5098. doi: 10.4049/jimmunol.178.8.5086.
- Palucka, A. K. and Coussens, L. M. (2016) 'The Basis of Oncoimmunology', *Cell*. Elsevier Inc., 164(6), pp. 1233–1247. doi: 10.1016/j.cell.2016.01.049.
- Papiernik, M. *et al.* (1998) 'Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency', *International Immunology*, 10(4), pp. 371–378. doi: 10.1093/intimm/10.4.371.
- Passarelli, A. *et al.* (2017) 'Immune system and melanoma biology: a balance between immunosurveillance and immune escape', *Oncotarget*, 8(62), pp. 106132–106142. doi: 10.18632/oncotarget.22190.
- Pedroza-Pacheco, I., Madrigal, A. and Saudemont, A. (2013) 'Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy', *Cellular & Molecular Immunology*. Nature Publishing Group, 10(3), pp. 222–229. doi: 10.1038/cmi.2013.2.
- Pickup, M., Novitskiy, S. and Moses, H. L. (2013) 'The roles of TGFβ in the tumour microenvironment', *Nature Reviews Cancer*. Nature Publishing Group, 13(11), pp. 788–799. doi: 10.1038/nrc3603.
- Plitas, G. *et al.* (2016) 'Regulatory T Cells Exhibit Distinct Features in Human Breast Cancer', *Immunity*. Elsevier Inc., 45(5), pp. 1122–1134. doi: 10.1016/j.immuni.2016.10.032.
- Quail, D. F. and Joyce, J. A. (2013) 'Microenvironmental regulation of tumor progression and metastasis', *Nature Medicine*, 19(11), pp. 1423–1437. doi: 10.1038/nm.3394.

- Read, S., Malmström, V. and Powrie, F. (2000) 'Cytotoxic T Lymphocyte–Associated Antigen 4 Plays an Essential Role in the Function of CD25 + CD4 + Regulatory Cells That Control Intestinal Inflammation', *The Journal of Experimental Medicine*, 192(2), pp. 295–302. doi: 10.1084/jem.192.2.295.
- Rosanò, L. *et al.* (2004) 'Endothelin-B receptor blockade inhibits molecular effectors of melanoma cell progression.', *Journal of cardiovascular pharmacology*, 44(November), pp. S136–S139. doi: 10.1097/01.fjc.0000166247.35992.dd.
- Saenger, Y. M. and Wolchok, J. D. (2008) 'The heterogeneity of the kinetics of response to ipilimumab in metastatic melanoma: Patient cases', *Cancer Immunity*, 8(October 2007), pp. 1–7. doi: 080102 [pii].
- Sakaguchi, S. *et al.* (1995) 'Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.', *The Journal of Immunology*, 155(3), p. 1151 LP-1164. Available at: <http://www.jimmunol.org/content/155/3/1151.abstract>.
- Sato, E. *et al.* (2005) 'Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer', *Proceedings of the National Academy of Sciences*, 102(51), pp. 18538–18543. doi: 10.1073/pnas.0509182102.
- Sato, T. *et al.* (2011) 'Interleukin 10 in the tumor microenvironment: A target for anticancer immunotherapy', *Immunologic Research*, 51(2–3), pp. 170–182. doi: 10.1007/s12026-011-8262-6.
- Sayour, E. J. *et al.* (2015) 'Increased proportion of FoxP3+ regulatory T cells in tumor infiltrating lymphocytes is associated with tumor recurrence and reduced survival in patients with glioblastoma', *Cancer Immunology, Immunotherapy*, 64(4), pp. 419–427. doi: 10.1007/s00262-014-1651-7.
- Schadendorf, D. *et al.* (2015) 'Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma', *Journal of Clinical Oncology*, 33(17), pp. 1889–1894. doi: 10.1200/JCO.2014.56.2736.
- Schreiber, R. D., Old, L. J. and Smyth, M. J. (2011) 'Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion', *Science*, 331(6024), pp. 1565–1570. doi: 10.1126/science.1203486.
- Shabaneh, T. B. *et al.* (2018) 'Oncogenic BRAFV600E Governs Regulatory T-cell Recruitment during melanoma tumorigenesis', *Cancer Research*, 78(17), pp. 5038–5049. doi: 10.1158/0008-5472.CAN-18-0365.
- Shimizu, J. *et al.* (1999) 'Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity.', *The Journal of Immunology*, 163(10), pp. 5211–8. doi: ji_v163n10p5211 [pii].

- Simpson, T. R. *et al.* (2013) 'Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma', *The Journal of Experimental Medicine*, 210(9), pp. 1695–1710. doi: 10.1084/jem.20130579.
- Smith, M. P. *et al.* (2017) 'Targeting endothelin receptor signalling overcomes heterogeneity driven therapy failure', *EMBO Molecular Medicine*, 9(8), pp. 1011–1029. doi: 10.15252/emmm.
- Soldano, S. *et al.* (2016) 'Alternatively Activated (M2) Macrophage Phenotype Is Inducible by Endothelin-1 in Cultured Human Macrophages', *PLOS ONE*. Edited by G. Zissel, 11(11), p. e0166433. doi: 10.1371/journal.pone.0166433.
- Suri-Payer, E. *et al.* (1998) 'CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells.', *J Immunol.*, 160(3), pp. 1212–8. doi: 10.4049/jimmunol.174.3.1446.
- Takeuchi, Y. and Nishikawa, H. (2016) 'Roles of regulatory T cells in cancer immunity', *International Immunology*, 28(8), pp. 401–409. doi: 10.1093/intimm/dxw025.
- Tanaka, K. *et al.* (2014) 'Endothelin regulates function of IL-17-producing T cell subset', *Life Sciences*. Elsevier Inc., 118(2), pp. 244–247. doi: 10.1016/j.lfs.2014.01.084.
- Tang, M. *et al.* (2015) 'Prognostic significance of in situ and plasma levels of transforming growth factor β 1, -2 and -3 in cutaneous melanoma', *Molecular Medicine Reports*, 11(6), pp. 4508–4512. doi: 10.3892/mmr.2015.3250.
- Tang, Y. *et al.* (2014) 'An Increased Abundance of Tumor-Infiltrating Regulatory T Cells Is Correlated with the Progression and Prognosis of Pancreatic Ductal Adenocarcinoma', *PLoS ONE*. Edited by H. Shiku, 9(3), p. e91551. doi: 10.1371/journal.pone.0091551.
- Tao, H. *et al.* (2012) 'Prognostic potential of FOXP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells', *Lung Cancer*. Elsevier Ireland Ltd, 75(1), pp. 95–101. doi: 10.1016/j.lungcan.2011.06.002.
- Thornton, A. M. and Shevach, E. M. (1998) 'CD4 + CD25 + Immunoregulatory T Cells Suppress Polyclonal T Cell Activation In Vitro by Inhibiting Interleukin 2 Production', *The Journal of Experimental Medicine*. Elsevier, 188(2), pp. 287–296. doi: 10.1084/jem.188.2.287.
- Tirosh, I. *et al.* (2016) 'Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq.', *Science*, 352(6282), pp. 189–96. doi: 10.1126/science.aad0501.

- Togashi, Y., Shitara, K. and Nishikawa, H. (2019) 'Regulatory T cells in cancer immunosuppression — implications for anticancer therapy', *Nature Reviews Clinical Oncology*. Springer US. doi: 10.1038/s41571-019-0175-7.
- Turnis, M. E. *et al.* (2016) 'Interleukin-35 Limits Anti-Tumor Immunity', *Immunity*. Elsevier Inc., 44(2), pp. 316–329. doi: 10.1016/j.immuni.2016.01.013.
- Umansky, V. and Sevko, A. (2012) 'Melanoma-induced immunosuppression and its neutralization', *Seminars in Cancer Biology*. Elsevier Ltd, 22(4), pp. 319–326. doi: 10.1016/j.semcancer.2012.02.003.
- Vence, L. *et al.* (2007) 'Circulating tumor antigen-specific regulatory T cells in patients with metastatic melanoma', *Proceedings of the National Academy of Sciences*, 104(52), pp. 20884–20889. doi: 10.1073/pnas.0710557105.
- Vignali, D. A. A., Collison, L. W. and Workman, C. J. (2008) 'How regulatory T cells work', *Nature Reviews Immunology*, 8(7), pp. 523–532. doi: 10.1038/nri2343.
- Wang, C., Lee, J. H. and Kim, C. H. (2012) 'Optimal Population of FoxP3+ T Cells in Tumors Requires an Antigen Priming-Dependent Trafficking Receptor Switch', *PLoS ONE*. Edited by F. Dieli, 7(1), p. e30793. doi: 10.1371/journal.pone.0030793.
- Wang, J. *et al.* (2017) 'UV-induced somatic mutations elicit a functional T cell response in the YUMMER1.7 Mouse Melanoma Model', *Pigment Cell & Melanoma Research*, 30(4), pp. 428–435. doi: 10.1111/pcmr.12591.
- Wing, K. *et al.* (2008) 'CTLA-4 Control over Foxp3+ Regulatory T Cell Function', *Science*, 322(5899), pp. 271–275. doi: 10.1126/science.1160062.
- Wouters, J. *et al.* (2015) 'First-in-Human Proof-of-Concept Study: Intralesional Administration of BQ788, an Endothelin Receptor B Antagonist, to Melanoma Skin Metastases', *The Oncologist*, 20(10), pp. 1121–1122. doi: 10.1634/theoncologist.2015-0139.
- Yamaguchi, T. and Sakaguchi, S. (2006) 'Regulatory T cells in immune surveillance and treatment of cancer', *Seminars in Cancer Biology*, 16(2), pp. 115–123. doi: 10.1016/j.semcancer.2005.11.005.
- Yi, Y. *et al.* (2013) 'The functional impairment of HCC-infiltrating $\gamma\delta$ T cells, partially mediated by regulatory T cells in a TGF β - and IL-10-dependent manner', *Journal of Hepatology*. European Association for the Study of the Liver, 58(5), pp. 977–983. doi: 10.1016/j.jhep.2012.12.015.
- Zhang, Y. *et al.* (2013) 'Genome-wide DNA methylation analysis identifies hypomethylated genes regulated by FOXP3 in human regulatory T cells', *Blood*, 122(16), pp. 2823–2836. doi: 10.1182/blood-2013-02-481788.
- Zheng, Y. *et al.* (2007) 'Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells', *Nature*, 445(7130), pp. 936–940. doi: 10.1038/nature05563.

Zou, W. and Restifo, N. P. (2010) 'TH17 cells in tumour immunity and immunotherapy', *Nature Reviews Immunology*. Nature Publishing Group, 10(4), pp. 248–256. doi: 10.1038/nri2742.

CHAPTER

4. Conclusions, Implications and Future Directions

4.1. Conclusions and Implications

Melanoma is the deadliest type of skin cancer accounting for 75% of all deaths related to cutaneous tumors (Schadendorf et al., 2015). The high mortality rate associated with melanoma is due to its remarkable metastatic potential (Damsky, Theodosakis and Bosenberg, 2014). Many cytokines are associated with melanoma progression, among them are the endothelins. Endothelins are cytokines ubiquitously expressed in the microenvironment of several tumors (Nelson et al., 2003). In carcinomas and glioblastoma the components of endothelin signaling are expressed by different cell types within TME (Aubert and Juillerat-Jeanneret, 2016). In melanoma is not clear whether stromal cells respond to the endothelin present in the microenvironment.

The present study corroborated previous findings (Demunter et al., 2001; Tang et al., 2008) demonstrating that Edn3/Ednrb signaling activation in melanoma increases tumor aggressiveness. In this study I showed that activation of endothelin signaling mediated aggressiveness of a broad range of melanomas, including BRAF^{V600E} and BRAF wild-type melanomas. Few melanoma cells expressed Ednrb in the YUMM1.7-GFP melanoma microenvironment suggesting an environmental role for endothelin signaling in melanoma microenvironment. The present study showed for the first time that Ednrb is expressed in several cell populations inside melanoma microenvironment. The clinical relevance of this finding was validated using publicly available RNA-seq data from melanoma patients (Tirosh et al., 2016). To a certain extent, Ednrb was expressed in both pro- and anti-tumorigenic cells indicating that endothelin activation might have

multiple effects in melanoma microenvironment. Since *K5-Edn3* tumors exhibited a more aggressive phenotype it is possible that in Edn3-rich microenvironment the net result benefited pro-tumorigenic cells. To date, no other study has shown Ednrb expression in intratumoral Tregs. Treg was the only population that was numerically different when *K5-Edn3* tumors were compared to wild-type tumors. This data suggests that when Edn3 is present in melanoma microenvironment there is an enrichment of intratumoral Tregs. Tumor-infiltrating Tregs suppress anti-tumor immunity (Nishikawa and Sakaguchi, 2010). Therefore, it is reasonable to claim that in abundance of Edn3 Tregs create an immunosuppressive environment in melanoma tumors granting escape from immunosurveillance.

Melanoma is recognized as one of the most immunogenic types of tumors (Passarelli et al., 2017). Melanoma also develops an immunosuppressive microenvironment resulting in evasion from immune detection and ultimately metastasis formation (Umansky and Sevko, 2012). Endothelins are commonly considered pro-inflammatory cytokines given their stimulatory roles during inflammatory response (Nett et al., 2006). Over the past years, mounting data have challenged the paradigm that endothelin solely stimulates inflammatory response. The present study supports the notion that endothelin promotes the formation of an immunosuppressive milieu in melanoma facilitating escape from tumor immunity. Endothelin was required for immune escape of highly immunogenic melanoma cells YUMMER1.7. Tregs orchestrated immunosuppression in YUMM1.7-GFP melanoma tumors. FOXP3 is considered the master regulator of Tregs suppressive functions (Fontenot, Gavin and Rudensky, 2003; Hori, Nomura

and Sakaguchi, 2003). FOXP3 was remarkably upregulated in *K5-Edn3* tumors confirming that Edn3 overexpression in melanoma microenvironment is associated with Treg enrichment. Tregs disrupt immune response against tumor through the secretion of immunosuppressive cytokines TGF- β and IL-10 (Yi et al., 2013). Accordingly, *K5-Edn3* tumors exhibited high levels of TGF- β and IL-10. This observation suggested that in presence of Edn3, the immunosuppressive features of Tregs are enhanced. Indeed, activation of endothelin signaling in Tregs prevented the expansion of CTLs possibly via GZB-mediated cytotoxicity. These data indicated that the response of Tregs to endothelin impaired immune response against melanoma. Unexpectedly, expression of chemokines and inflammatory cytokines were downregulated in *K5-Edn3* tumors. Cytokines that elicit anti-tumor immunity, namely IL-17E and IL-28 (Numasaki *et al.*, 2007; Zou and Restifo, 2010), exhibited reduced levels in *K5-Edn3* tumors. This result endorsed the idea that endothelin mitigated tumor immunity in YUMM1.7-GFP melanoma tumors. Importantly, this is the first report to associate overexpression of endothelin with reduction of IL-28 production. YUMM1.7-GFP tumors exposed to high levels of Edn3 were sensitive to immune checkpoint inhibitor (anti-CTLA-4) as well as to Ednrb blockage (BQ-788), although the response to BQ-788 was more pronounced.

Metastatic melanoma has a very poor prognosis with the 1-year overall survival rate around 25% (Korn *et al.*, 2008). Although YUMM1.7-GFP tumors responded to high levels of Edn3 by growing larger, few metastatic lesions were observed, and only in *K5-Edn3* mice. YUMM1.7 cells mirror the genetic alterations commonly

found in human melanomas, namely *BRAF*^{V600E/+}; *Pten*^{-/-}; *Cdkn2a*^{-/-} mutations, but were shown to have limited metastatic features (Meeth *et al.*, 2016). Therefore, the findings from the present study should be viewed with caution regarding melanoma metastasis.

The anti-metastatic potential of immune checkpoint and Ednrb inhibitors was not assessed in the current study. Still, YUMM1.7-GFP tumors exposed to high levels of Edn3 were sensitive to immune checkpoint inhibitor and Ednrb blockage as both treatments significantly decreased tumor growth. Increased tumor volume is correlated with elevated likelihood of melanoma dissemination to distant organs (Walton *et al.*, 2015). Thus, it is plausible that, in the context of Edn3 overexpression, immune checkpoint inhibitor as well as Ednrb blockage may have prevented melanoma metastasis formation.

Altogether the data in the present study advocate for an environmental role of Edn3/Ednrb signaling in melanoma. Endothelin mediates immunosuppression in melanoma microenvironment resulting in escape from tumor immunity. These findings are summarized in Figure 4.1. The present study endorses the framework that endothelin signaling may have multiple effects in the melanoma microenvironment given that melanoma cells and different populations of stromal cells expressed Ednrb in the YUMM1.7-GFP tumors. Melanoma cells may be directly affected by an Edn3-rich microenvironment. It has already been shown that melanoma cells express EDNRB (Asundi *et al.*, 2011; Bagnato *et al.*, 2004b; Lahav, Heffner, & Patterson, 1999; Yohn *et al.*, 1994). Activation of EDNRB on

melanoma cells triggers many cellular events relevant for tumor progression such as proliferation, migration, and invasion of melanoma cells (Bagnato et al., 2004a). Endothelins are also secreted by melanoma cells (Chiriboga et al., 2016; Tang et al., 2008). In the context of Edn3 overexpression, it is possible that the autocrine activation of Ednrb in melanoma cells enhanced the abovementioned cellular events.

Abundance of Edn3 in the melanoma microenvironment might also have indirectly favored melanoma cells. Activation of endothelin signaling in fibroblasts induces secretion of ECM components and metalloproteinases (Knowles *et al.*, 2012), which might have created a permissive stroma for melanoma cell growth and invasion. Endothelin signaling controls angiogenic events in endothelial cells (Salani, Taraboletti, *et al.*, 2000). New blood vessels increase nutrient supply to tumor cells as well as create new routes for metastatic cell dispersion (Smith and Kang, 2013). YUMM1.7-GFP melanomas from both wild-type and *K5-Edn3* mice exhibited anti- and pro-tumorigenic immune cells expressing Ednrb, which indicates potential opposite functions of endothelin signaling in the melanoma microenvironment. Given that *K5-Edn3* tumors have a more aggressive phenotype, it is reasonable to suggest that an Edn3-rich microenvironment favors the effect of endothelin in pro-tumorigenic cells. The present study explored the suppressive role of Edn3 in the melanoma microenvironment. The context of impaired tumor immunity seems to benefit melanoma cells given that anti-tumor cells might become unable to recognize and kill cancer cells.

4.2. Future Directions

The present study proposes that melanoma should be added to the roster of tumor types in which activation of endothelin signaling has a microenvironmental role. Ultra-violet light (UV) exposure is considered the major risk factor for melanoma (Lawrence et al., 2013). Interestingly, keratinocytes release endothelin upon UV light exposure (Imokawas, Yada and Miyagishi, 1992). In the *K5-Edn3* mouse model (Garcia *et al.*, 2008) overexpression of Edn3 is restricted to keratinocytes. Thus, *K5-Edn3* mice used in this study constitute a suitable system to explore the microenvironmental role of endothelin in melanoma. A different approach to validate the observations made using the *K5-Edn3* mouse model would be the injection of melanoma cells in mice that lack expression of Ednrb. The *piebald lethal* (*Ednrb^{sl/sl}*) mouse would be appropriate for further studies since it harbors spontaneous deletion in the *Ednrb* gene (Hosoda et al., 1994). Injection of YUMM1.7-GFP melanoma cells in *piebald lethal* mice would corroborate the microenvironmental role Edn3/Ednrb signaling in melanoma microenvironment. A similar strategy was employed to describe the stromal functions of Ednrb in breast cancer. Injection of breast cancer cells overexpressing Ednra generated tumors with diminished growth and metastatic potential in *Ednrb* deficient rat. Absence of stromal Ednrb impaired immune cells infiltration and cytokine production resulting in reduced tumor aggressiveness. Lack of Ednrb in TME decreased the recruitment of TAMs and the amount of pro-inflammatory cytokine TNF- α (Binder et al., 2009).

The present study suggested that endothelin promotes evasion from tumor immunity in YUMMER1.7 melanomas. It has been demonstrated that Tregs prevents the regression of YUMMER1.7 tumors (Wang *et al.*, 2017). It was not clear whether activation of Ednrb in Tregs mediated the impaired immunosurveillance in YUMMER1.7 tumors from *K5-Edn3* mice. Flow cytometry experiments using Ednrb and CD25 antibodies should be performed in *K5-Edn3* tumors to test if activation of endothelin signaling in Tregs plays a role during YUMMER1.7 immune escape. Additionally, FOXP3 expression at mRNA and proteic level should be carried out in *K5-Edn3* tumors to confirm that endothelin is required for Treg suppressive functions in YUMMER1.7 melanomas.

The current data implicated that overexpression of Edn3 in melanoma microenvironment results in intratumoral Tregs enrichment. Still, the mechanism responsible for Tregs accumulation in *K5-Edn3* tumors was not explored. It is possible that endothelin impacted Tregs proliferation, recruitment or conversion of conventional T cells into Tregs (Lee, 2017). In order to clarify the role of Ednrb activation in Tregs, suppressive cells should be isolated from tumors using Treg isolation kit (MACS Miltenyi Biotec, 130-091-041) and treated with Edn3. Treg proliferation can be addressed using CFSE dilution. The migratory effect of Tregs in response to endothelin could be assessed using Boyden chamber assay. To test if endothelin mediates the conversion of conventional T cells into Tregs isolated naïve CD4⁺ T cell should be cultivated in presence of Edn3. It is possible that isolation of intratumoral Tregs using Treg isolation kit yield a small number of cells. Alternatively, mouse spleen can be use as Treg source.

Production of GZB by intratumoral Tregs suppresses the anti-tumor response of NKs and CTLs (Cao *et al.*, 2007). Nevertheless, GZB is also expressed by CTLs and NKs (Lieberman, 2003). Due to technical limitations, it was not possible to demonstrate that Tregs accounted for augmented expression of GZB in *K5-Edn3* tumors. In order to elucidate whether activation of endothelin signaling triggers GZB expression in Tregs, isolated Tregs should be cultured in presence of Edn3 and GZB expression measured using qRT-PCR and Western Blotting.

Preclinical studies have shown that Ednrb inhibition effectively reduced melanoma growth (Lahav, Heffner and Patterson, 1999; Spinella *et al.*, 2007; Smith *et al.*, 2017). The clinical relevance of EDNRB blockage is still elusive (Kefford *et al.*, 2007; Wouters *et al.*, 2015). This is probably due to the lack of previous screening for patients with high levels of EDNRB. Immunotherapy approaches using immune checkpoint inhibitors have reached exciting results in patients with melanoma. Yet, some patients do not respond to immunotherapies and new strategies are imperatively required (Binnewies *et al.*, 2018). The data from the present study indicate that Ednrb blockage might improve the outcome of immunotherapies. Indeed, EDNRB blockage enhanced the results of immunotherapy in a preclinical study of ovarian cancer (Buckanovich *et al.*, 2008). Additional studies using large cohorts should be carried out to validate the successful preliminary results of Ednrb inhibitor treatment in *K5-Edn3* mice bearing YUMM1.7-GFP melanomas. Further, clinical trials aiming to block endothelin signaling might be performed in melanoma patients that express high levels of EDNRB and are under ipilimumab treatment.

4.3. Figure

Figure 4.1 Microenvironmental role of Edn3/Ednrb signaling in melanoma

Several cell types are present in the microenvironment of melanomas that express endogenous levels of Edn3. In these tumors there is reduced expression on immunosuppressive cytokines IL-10 and TGF-β as well as GZB. Chemokines and cytokines that elicit anti-tumor immunity are highly expressed in melanomas that exhibit endogenous levels of Edn3. Conversely, melanomas growing in Edn3-rich microenvironment exhibit enrichment of Ednrb⁺ Tregs. Endothelin promotes the formation of an immunosuppressive milieu characterized by augmented expression of immunosuppressive cytokines IL-10 and TGF-β as well as GZB and diminished levels of inflammatory cytokines and chemokines.

4.4. References

- Asundi, J. *et al.* (2011) 'An Antibody-Drug Conjugate Targeting the Endothelin B Receptor for the Treatment of Melanoma', *Clinical Cancer Research*, 17(5), pp. 965–975. doi: 10.1158/1078-0432.CCR-10-2340.
- Aubert, J. D. and Juillerat-Jeanneret, L. (2016) 'Endothelin-Receptor Antagonists beyond Pulmonary Arterial Hypertension: Cancer and Fibrosis', *Journal of Medicinal Chemistry*, 59(18), pp. 8168–8188. doi: 10.1021/acs.jmedchem.5b01781.
- Bagnato, A. *et al.* (2004a) 'Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression', *Cancer Res*, 64(4), pp. 1436–1443.
- Bagnato, A. *et al.* (2004b) 'Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions in Melanoma Cell Progression', *Cancer research*, 64(4), pp. 1436–1443.
- Binder, C. *et al.* (2009) 'Stromal endothelin B receptor-deficiency inhibits breast cancer growth and metastasis', *Molecular Cancer Therapeutics*, 8(8), pp. 2452–2460. doi: 10.1158/1535-7163.MCT-09-0032.
- Binnewies, M. *et al.* (2018) 'Understanding the tumor immune microenvironment (TIME) for effective therapy', *Nature Medicine*. Springer US, 24(5), pp. 541–550. doi: 10.1038/s41591-018-0014-x.
- Buckanovich, R. J. *et al.* (2008) 'Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy.', *Nature medicine*, 14(1), pp. 28–36. doi: 10.1038/nm1699.
- Cao, X. *et al.* (2007) 'Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance', *Immunity*, 27(4), pp. 635–646. doi: 10.1016/j.immuni.2007.08.014.
- Chiriboga, L. *et al.* (2016) 'Endothelin-1 in the tumor microenvironment correlates with melanoma invasion', *Melanoma Research*, 26(3), pp. 236–44. doi: 10.1097/CMR.0000000000000235.
- Damsky, W. E., Theodosakis, N. and Bosenberg, M. (2014) 'Melanoma metastasis: new concepts and evolving paradigms.', *Oncogene*. Nature Publishing Group, 33(19), pp. 2413–22. doi: 10.1038/onc.2013.194.
- Demunter, A. *et al.* (2001) 'Expression of the endothelin-B receptor in pigment cell lesions of the skin: Evidence for its role as tumor progression marker in malignant melanoma', *Virchows Archiv*, 438(5), pp. 485–491. doi: 10.1007/s004280000362.
- Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003) 'Foxp3 programs the development and function of CD4+CD25+ regulatory T cells', *Nature Immunology*, 4(4), pp. 330–336. doi: 10.1038/ni904.

- Garcia, R. J. *et al.* (2008) 'Endothelin 3 Induces Skin Pigmentation in a Keratin-Driven Inducible Mouse Model', *Journal of Investigative Dermatology*, 128(1), pp. 131–142. doi: 10.1038/sj.jid.5700948.
- Hori, S., Nomura, T. and Sakaguchi, S. (2003) 'Control of Regulatory T Cell Development by the Transcription Factor Foxp3', *Science*, 299(5609), pp. 1057–1061. doi: 10.1126/science.1079490.
- Hosoda, K. *et al.* (1994) 'Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice', *Cell*, 79(7), pp. 1267–1276. doi: 10.1016/0092-8674(94)90017-5.
- Imokawas, G., Yada, Y. and Miyagishi, M. (1992) 'Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes', *J Biol Chem*, 267(34), pp. 24675–24680.
- Kefford, R. *et al.* (2007) 'A Phase II study of bosentan, a dual endothelin receptor antagonist, as monotherapy in patients with stage IV metastatic melanoma', *Investigational New Drugs*, 25(3), pp. 247–252. doi: 10.1007/s10637-006-9014-7.
- Knowles, J. P. *et al.* (2012) 'Endothelin-1 stimulates colon cancer adjacent fibroblasts', *International Journal of Cancer*, 130(6), pp. 1264–1272. doi: 10.1002/ijc.26090.
- Korn, E. L. *et al.* (2008) 'Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials', *Journal of Clinical Oncology*, 26(4), pp. 527–534. doi: 10.1200/JCO.2007.12.7837.
- Lahav, R., Heffner, G. and Patterson, P. H. (1999) 'An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(20), pp. 11496–11500.
- Lawrence, M. S. *et al.* (2013) 'Mutational heterogeneity in cancer and the search for new cancer-associated genes', *Nature*, 499(7457), pp. 214–218. doi: 10.1038/nature12213.
- Lee, G. R. (2017) 'Phenotypic and Functional Properties of Tumor-Infiltrating Regulatory T Cells', *Mediators of inflammation*, 2017, pp. 1–9. doi: 10.1155/2017/5458178.
- Lieberman, J. (2003) 'The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal', *Nature Reviews Immunology*, 3(5), pp. 361–370. doi: 10.1038/nri1083.
- Meeth, K. *et al.* (2016) 'The YUMM lines: a series of congenic mouse melanoma cell lines with defined genetic alterations', *Pigment Cell & Melanoma Research*, 29(5), pp. 590–7. doi: 10.1111/pcmr.12498.

- Nelson, J. *et al.* (2003) 'The endothelin axis: Emerging role in cancer', *Nature Reviews Cancer*, 3(2), pp. 110–116. doi: 10.1038/nrc990.
- Nett, P. C. *et al.* (2006) 'Recent developments on endothelin antagonists as immunomodulatory drugs--from infection to transplantation medicine.', *Recent patents on cardiovascular drug discovery*, 1(3), pp. 265–276. doi: 10.2174/157489006778776990.
- Nishikawa, H. and Sakaguchi, S. (2010) 'Regulatory T cells in tumor immunity', *International Journal of Cancer*, 127(4), pp. 759–767. doi: 10.1002/ijc.25429.
- Numasaki, M. *et al.* (2007) 'IL-28 Elicits Antitumor Responses against Murine Fibrosarcoma', *The Journal of Immunology*, 178(8), pp. 5086–5098. doi: 10.4049/jimmunol.178.8.5086.
- Passarelli, A. *et al.* (2017) 'Immune system and melanoma biology: a balance between immunosurveillance and immune escape', *Oncotarget*, 8(62), pp. 106132–106142. doi: 10.18632/oncotarget.22190.
- Salani, D. *et al.* (2000) 'Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo', *American Journal of Pathology*, 157(5), pp. 1703–1711. doi: 10.1016/S0002-9440(10)64807-9.
- Schadendorf, D. *et al.* (2015) 'Melanoma', *Nature Reviews Disease Primers*, 1(15003). doi: 10.1038/nrdp.2015.3.
- Smith, H. A. and Kang, Y. (2013) 'The Metastasis-Promoting Roles of Tumor-Associated Immune Cells', *Journal of Molecular Medicine*, 91(4), pp. 411–429. doi: 10.1007/s00109-013-1021-5.
- Smith, M. P. *et al.* (2017) 'Targeting endothelin receptor signalling overcomes heterogeneity driven therapy failure', *EMBO Molecular Medicine*, 9(8), pp. 1011–1029. doi: 10.15252/emmm.
- Spinella, F. *et al.* (2007) 'Endothelin-1 and endothelin-3 promote invasive behavior via hypoxia-inducible factor-1 α in human melanoma cells', *Cancer Research*, 67(4), pp. 1725–1734. doi: 10.1158/0008-5472.CAN-06-2606.
- Tang, L. *et al.* (2008) 'Endothelin-3 is produced by metastatic melanoma cells and promotes melanoma cell survival', *J Cutan Med Surg*, 12(2), pp. 64–70. doi: 10.2310/7750.2008.06164.
- Tirosh, I. *et al.* (2016) 'Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq.', *Science*, 352(6282), pp. 189–96. doi: 10.1126/science.aad0501.
- Umansky, V. and Sevko, A. (2012) 'Melanoma-induced immunosuppression and its neutralization', *Seminars in Cancer Biology*. Elsevier Ltd, 22(4), pp. 319–326. doi: 10.1016/j.semcancer.2012.02.003.

- Walton, R. *et al.* (2015) 'Tumor volume: an adjunct prognostic factor in cutaneous melanoma', *Cutis*, 94(5), pp. 226–30.
- Wang, J. *et al.* (2017) 'UV-induced somatic mutations elicit a functional T cell response in the YUMMER1.7 Mouse Melanoma Model', *Pigment Cell & Melanoma Research*, 30(4), pp. 428–435. doi: 10.1111/pcmr.12591.
- Wouters, J. *et al.* (2015) 'First-in-Human Proof-of-Concept Study: Intralesional Administration of BQ788 , an Endothelin Receptor B Antagonist , to Melanoma Skin Metastases', *The Oncologist*, 9(69), pp. 1121–1122. doi: 10.1634/theoncologist.2015-0139.
- Yi, Y. *et al.* (2013) 'The functional impairment of HCC-infiltrating $\gamma\delta$ T cells, partially mediated by regulatory T cells in a TGF β - and IL-10-dependent manner', *Journal of Hepatology*. European Association for the Study of the Liver, 58(5), pp. 977–983. doi: 10.1016/j.jhep.2012.12.015.
- Yohn, J. J. *et al.* (1994) 'Human Melanoma Cells Express Functional Endothelin-1 Receptors', *Biochemical and Biophysical Research Communications*, 201(1), pp. 449–457. doi: 10.1006/bbrc.1994.1722.
- Zou, W. and Restifo, N. P. (2010) 'TH17 cells in tumour immunity and immunotherapy', *Nature Reviews Immunology*. Nature Publishing Group, 10(4), pp. 248–256. doi: 10.1038/nri2742.

VITA

JULIANO FREITAS

EDUCATION AND EXPERIENCE

2006-2012	Bachelor's Degree Biological Science Federal University of Viçosa (UFV), Viçosa, MG, Brazil
2011	Internship Equipe Génétique du Développement des Mélanocytes, Institut Curie, Orsay, France
2012-2016	Master <i>en route</i> to PhD Department of Biological Sciences Florida International University, Miami, FL, USA
2012-2017	Graduate Teaching Assistant Department of Biological Sciences Florida International University, Miami, FL, USA
2017-2019	Graduate Research Assistant Department of Biological Sciences Florida International University, Miami, FL, USA
2014-2019	PhD Candidate Department of Biological Sciences Florida International University, Miami, FL, USA
2015	BRI Summer Research Award Florida International University, Miami, FL, USA
2016	Doctoral Evidence Acquisition (DEA) Fellowship Florida International University, Miami, FL, USA
2017	Young Investigator Award Fifteenth International Conference on Endothelin Prague, Czech Republic

SELECTED PUBLICATIONS AND PRESENTATIONS

Freitas, J., Kos, L. Endothelin 3 as Main Regulator of Lung and Brain Premetastatic Niche Formation in Melanoma. From Melanocyte Development to Melanoma Therapies - Basic Science and Clinical Applications, Reykjavik, Iceland, June 2015 (Oral Presentation)

Freitas, J., Kos, L. Evaluation of premetastatic niche formation in a mouse model of spontaneous melanoma lung metastasis. Society for Melanoma Research Congress, November 18-21, 2015, San Francisco, California (Poster Presentation)

Freitas, J., Cavallini, M., Palmer, J., Kos, L. The Role of Endothelin 3 during Melanoma Lung Premetastatic Niche Formation. 20th Conference of the PanAmerican Society for Pigment Cell Research (PASPCR) October 5-8, 2016, Baltimore, Maryland (Poster Presentation)

Freitas, J., Cavallini, M., Palmer, J., Kos, L. The Role of Endothelin 3 during Melanoma Lung Premetastatic Niche Formation. Society for Melanoma Research Congress, November 6-9, 2016, Boston, Massachusetts (Poster Presentation)

Freitas, J., Leng, F., Kos, L. Netropsin Blocks EMT of murine BRAF mutated melanoma cells. American Association of Cancer Research (AACR) Meeting, April 1-5, 2017, Washington, District of Columbia (Poster Presentation)

Freitas, J., Cavallini, M., Lopez, J., Palmer, J., Kos, L. The non-cell autonomous role of Edn3/Ednrb signaling during melanoma lung metastasis formation. IPCC, Denver, Colorado, August 2017 (Oral Presentation)

Freitas, J., Cavallini, M., Lopez, J., Palmer, J., Kos, L. The non-cell autonomous role of Edn3/Ednrb signaling during melanoma lung metastasis formation. The Fifteenth International Conference on Endothelin, Prague, Czech Republic, October 2017 (Oral Presentation)

Freitas, J., Lopez, J., Cavallini, M., Llorian, C., Kos, L. The immunosuppressive role of Edn3/Ednrb signaling in the melanoma microenvironment. Society for Melanoma Research Congress, October 24-27, 2018, Manchester, England (Poster Presentation)