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DE ROL VAN BNIP3 IN HET MODULEREN VAN DE INTERACTIE TUSSEN MELANOMACELLEN EN IMMUUNCELLEN

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Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Biomedische Wetenschappen

Juni, 2018

KU Leuven
Biomedical Sciences Group
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BNIP3 AND ITS ROLE IN MODULATING THE INTERFACE BETWEEN MELANOMA CELLS AND IMMUNE CELLS

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Science.

June, 2018

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Acknowledgements

In September 2016, my supervisor Patrizia Agostinis gave me the opportunity to take part to the conference "Cell Symposia: 100 years of Phagocytosis", that took place in the gorgeous Sicily. You can easily deduce that I am extremely grateful to her for that chance she gave me and, for several reasons. To mention a few: fascinating topic, remarkable speakers, 3 Nobel Prizes present, extraordinary location (in my beloved country), stimulating and challenging discussions with colleagues and PIs. And the list could be longer. But to make a long story short, in a word, it was simply MAGNIFICENT!

I still have - and I will always have- good memories of that experience. But there is especially one that is still with me and I am sure, will accompany me for long time, probably for the rest of my life.

Among the scientists I had chance to discuss my data with, there was a PI in particular that left me an "interesting quote", if I can define it in this way. At a certain point of our discussion, this relatively young African guy told me: "You know, the PhD is not about how many techniques you learn or how many papers you publish. The PhD is rather a "school of life": it teaches and trains you to re-raise after every fall!" I must say, after almost 5-years of PhD that, "the Guy", was right!

And here I am, thanking all those that were with me during those "falls", giving me a hand to help me to raise up again. The list is really long and I will not mention all of them one by one, basically for two reasons. The first one is a matter of space (it would take me additionally 10 pages and I cannot take the liberty to exceed the limit allowed). Secondly and mainly, because each of this person (who will read this manuscript or will be present at my defence) perfectly knows the contribution she/he gave to me during this journey here in Leuven. So:

THANKS TO ALL!

To my parents, to my beloved sisters, to my brother-in-law and all the close members of my family: I will never have enough words of gratitude!

To all my friends, the far and the close, the “new” and the “old”, always there for me, no matter the distance and the situations.
To all my colleagues (the current and the past ones) for their understanding, support and above all discretion during the tough times. And of course for sharing the funny breaks and moments ;)
To my supervisor who supported my project and gave me the chance to reach this stage, independently of the circumstances.
And of course, I cannot forget the “discouraging” people: they gave me a really good reason NOT to GIVE UP!

Summary

The hypoxia responsive protein BNIP3 plays an important role in promoting cell death and/or autophagy, ultimately resulting in a cancer type-dependent, tumour-enhancer or tumour-suppressor activity. We previously reported that in melanoma cells, BNIP3 regulates cellular morphology, mitochondrial clearance, cellular viability and maintains protein expression of CD47, a pro-cancerous, immunosuppressive 'don't eat me' signal. Surface-exposed CD47 is often up-regulated by cancer cells to avoid clearance by phagocytes and to suppress ICD (**I**mmunogenic **C**ell **D**eath) elicited by anti-cancer therapies. However, whether melanoma-associated BNIP3 modulates CD47-associated immunological effects or ICD has not been properly explored. To this end, here we evaluated the impact of the genetic ablation of BNIP3 (i.e. BNIP3^{KD}) in melanoma cells, on macrophage-based phagocytosis, polarization and chemotaxis both *in vitro* and *in vivo*, by means of intra peritoneum (IP) injection in mice. We also assessed the ability of BNIP3 to affect the *in vivo* growth of B16-F10 melanoma cells transplanted in syngeneic immune-competent mice, as well as the polarization status of the recruited TAMs (**T**umour **A**ssociated **M**acrophage**s**). Additionally, we tested its effects on crucial determinants of chemotherapy-induced ICD (i.e. danger signals), as well as *in vivo* anti-cancer vaccination effect. Interestingly, loss of BNIP3 reduced the expression of CD47 in both normoxic and hypoxic conditions while macrophage-phagocytosis and -chemotaxis were accentuated only when BNIP3^{KD} melanoma cells were exposed to hypoxia. Furthermore, BNIP3-deficient tumours displayed a significantly delayed tumour growth compared to the BNIP3-proficient counterparts. Although loss of BNIP3 in B16-F10 melanoma impaired the recruitment of TAMs it did not affect their polarization. Finally, when exposed to the ICD inducer mitoxantrone, the loss of melanoma cell-associated BNIP3 did not alter apoptosis-induction, but significantly prevented ATP secretion and reduced phagocytic clearance of dying cells. In line with this, prophylactic vaccination experiments showed that the loss of BNIP3 tends to increase the intrinsic resistance of B16-F10 melanoma cells to ICD-associated anti-cancer vaccination effect *in vivo*. Thus, normoxic vs. hypoxic and alive vs. dying

cell contexts influence the ultimate immunomodulatory roles of melanoma cell-associated BNIP3.

Samenvatting

BNIP3 is een eiwit waarvan de expressie verhoogt na blootstelling aan hypoxie. Het kan celdood en/of autofagie bevorderen dat, afhankelijk van het kankertype, tumorprogressie ondersteunende ofwel onderdrukkende effecten bewerkstelligt. In voorgaand onderzoek hebben we gerapporteerd dat BNIP3 in melanoma cellen enkele cellulaire aspecten reguleert waaronder morfologie, mitochondriële afbraak, overleving en het behoud van CD47 expressie. CD47 is een pro-tumorigeen eiwit, mede toe te schrijven aan diens immuunonderdrukkend 'eet-me-niet' signaal. Daarom is CD47 expressie op het celoppervlak van kankercellen vaak verhoogd om zo te voorkomen dat zÿ onderworpen worden aan fagocytose en om ICD (immunogene celdood) te onderdrukken die kan worden uitgelokt door bepaalde antikankertherapieën. Desondanks is het nog onbekend of melanoma-geassocieerd BNIP3 een rol speelt in de CD47-afhankelijke effecten op het afweerssysteem of ICD. Om dit te bestuderen hebben we gebruik gemaakt van genetisch gemodificeerde melanoma cellen die BNIP3 verlaagd tot expressie brengen ($BNIP3^{KD}$). We hebben vastgesteld of een tekort aan BNIP3 een effect heeft op de macrofaag-gemedieerde fagocytose, macrofaag polarizatie en aantrekking *in vitro* alsook *in vivo* door middel van intraperitoneale (i.p.) injectie in muizen. Ook is bepaald of BNIP3 invloed heeft op de tumorgroei van B16-F10 melanomacellen die in syngene immuuncompetente muizen zijn geïnjecteerd en op depolarizatie van tumor-geïnfiltreerde macrofagen (zogeheten tumor-geassocieerde macrofagen [TAMs]). Daarenboven zijn effecten op bepalende factoren voor chemotherapie-geïnduceerde ICD en op de doeltreffendheid van antikanker vaccinatie geëvalueerd. Een belangrijke bevinding is dat $BNIP3^{KD}$ melanoma cellen een verlaagde expressie van CD47 onder zowel normoxische alsook hypoxische condities vertonen. Daarentegen is de macrofaag-gemedieerde fagocytose en aantrekking alleen versterkt wanneer $BNIP3^{KD}$ melanoma cellen aan hypoxie worden blootgesteld. Tumoren bestaande uit $BNIP3^{KD}$ melanoma cellen vertonen een vertraagde groei in vergelijking tot controle tumoren en, in $BNIP3^{KD}$ B16-F10 tumoren, een verlaagde TAM infiltratie. Desondanks was de polarizatie onveranderd. Indien blootgesteld aan mitoxantrone, dat ICD induceert, is de omvang van apoptose

onverandert in BNIP3^{KD} melanomas, maar is de ATP secretie aangedaan en is de fagocytische opruiming van dode cellen verlaagd. Overeenkomstig met deze bevindingen laten profylactische vaccinatie experimenten zien dat een tekort aan BNIP3 in B16-F10 melanomacellen een verhoogde intrinsieke weerstandigheid lijkt te bewerkstelligen tegen ICD-geassocieerde antikanker vaccinatie *in vivo*. Dus normoxische vs. hypoxische en levende vs. stervende condities bepalen het modulerende effect op het afweersysteem door melanoma-geassocieerd BNIP3.

List of abbreviations

ADM	**AD**reno**M**edullin
ATG	**A**u**T**opha**G**y related
ATP	**A**denosine 5′-**T**ri**P**hosphate
Bak	**B**cl-2-**a**ntagonist/**k**iller
Bax	**B**cl-2-**a**ssociated **x** protein
Bcl-2	**B**-**c**ell **l**eukaemia/**l**ymphoma-**2**
Bcl-X_L	**B**-**c**ell **l**ymphoma e**x**tra-**l**arge
(b)FGF	(**b**asic)**F**ibroblast **G**rowth **F**actor
BNIP3	**B**cl-2 [**B**-**c**ell **l**eukaemia/**l**ymphoma-**2**]/Adenovirus E1B1] **N**ineteen kD **I**nteracting **P**rotein **3**
CALR	**CALR**eticulin
CCL-	**C**-**C** motif **L**igand-
CD-	**C**luster of **D**ifferentiation-
CR	**C**omplement **R**eceptor
CXCL-	chemokine (**C**-**X**-**C** motif) **L**igand-
CX3CL-1	chemokine (**C**-**X3**-**C** motif) **L**igand-**1**
DAMPs	**D**amage-**A**ssociated **M**olecular **P**atterns
DCs	**D**endritic **C**ells
ECM	**E**xtra**C**ellular **M**atrix
EGF	**E**pidermal **G**rowth **F**actor
eIF2α	**e**ukaryotic **I**nitiation **F**actor **2** **α**
ER	**E**ndoplasmic **R**eticulum
ERK	**E**xtracellular-signal-**R**egulated **K**inase
FBS	**F**oetal **B**ovine **S**erum
FC	**F**low **C**ytometer
F_cR	**F**$_c$ **R**eceptors
FDA	**F**ood and **D**rug **A**dministration
GM-CSF	**G**ranulocyte **M**acrophage-**C**olony **S**timulating **F**actor
gp100	**g**lyco**p**rotein 100
HIF-1α	**H**ypoxia **I**nducible **F**actor-**1α**

HIF-2α	**H**ypoxia **I**nducible **F**actor-**2α**
HMGB1	**H**igh **M**obility **G**roup **B**ox **1**
Hyp	**Hyp**oxia
Hyp-PDT	**Hyp**ericin-based **P**hoto**D**ynamic **T**herapy
ICD	**I**mmunogenic **C**ell **D**eath
IDO-1	**I**ndoleamine 2,3-**D**i**O**xigenase-**1**
IFNs	**I**nter**F**ero**Ns**
IFN-β	**I**nter**F**ero**N**-**β**
IFN-γ	**I**nter**F**ero**N**-**γ**
IL-	**I**nter**L**eukin-
KD	**K**nock **D**own
LC3	Microtubule-associated protein 1A/1B-**L**ight **C**hain **3**
LIR	**L**C3-**I**nteracting **R**egion
LPS	**L**ipo**P**oly**S**accharides
MART-1	**M**elanoma-associated **A**ntigen **R**ecognized by **T** cells-**1**
MCP-1	**M**onocyte **C**hemoattractant **P**rotein-1
M-CSF	**M**acrophage-**C**olony **S**timulating **F**actor
MDSCs	**M**yeloid-**D**erived **S**uppressor **C**ell**s**
MEK	**M**APK/**E**RK **K**inase
MHC-I	**M**ajor **H**istocompatibility **C**omplex class-**I**
MHC-II	**M**ajor **H**istocompatibility **C**omplex class-**II**
MITF	**MI**crophthalmia-associated **T**ranscription **F**actor
MMPs	**M**atrix **M**etallo**P**rotease**s**
mPTP	**m**itochondrial **P**ermeability **T**ransition **P**ore
mtDNA	**m**i**t**ochondrial **DNA**
mTORC1	the **m**echanistic **T**arget **O**f **R**apamycin **C**omplex **1**
MTX	**M**i**T**o**X**antrone
NDV	**N**ewcastle **D**isease **V**irus
NF-κB	**N**uclear **F**actor *k*appa **B**eta
Nor	**Nor**moxia
PDGF	**P**latelet **D**erived **G**rowth **F**actor

PERK	protein kinase R (**P**KR)-like **E**ndoplasmic **R**eticulum **K**inase
PGE-2	**P**rosta**G**landin **E**-**2**
PINK-1	**P**TEN-**IN**duced putative **K**inase-**1**
PI3K	**P**hospho**I**nositide **3**-**K**inase
PLAGL-2	**PL**eomorphic **A**denomas **G**ene **L**ike-**2**
PTEN	**P**hosphatase and **TEN**sin homolog
QRT-PCR	**Q**uantitative **R**everse **T**ranscription-**P**olymerase **C**hain **R**eaction
Raf	**R**apidly **a**ccelerated **f**ibrosarcoma
Ras	**Ra**t **s**arcoma
RIP3	**R**eceptor-**I**nteracting **P**rotein kinase **3**
ROS	**R**eactive **O**xygen **S**pecies
RPMI	**R**oswell **P**ark **M**emorial **I**nstitute
RT	**R**oom **T**emperature
SHP-1	**S**rc **H**omology **P**hosphatases-**1**
SHP-2	**S**rc **H**omology **P**hosphatases-**2**
SIRPα	**SI**gnal **R**egulatory **P**rotein **α**
TAMs	**T**umour **A**ssociated **M**acrophage**s**
TGF-β	**T**ransforming **G**rowth **F**actor-**β**eta
Th1	**T** **h**elper (type) **1**
Th2	**T** **h**elper (type) **2**
TLRs	**T**oll-**L**ike **R**eceptor**s**
TME	**T**umour **M**icro**E**nvironment
TNF	**T**umour **N**ecrosis **F**actor
T_{regs}	**T** **reg**ulatory cell**s**
UV	**U**ltra**V**iolet
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor

1. Introduction

1.1.Melanoma: general overview and epidemiology

Cutaneous malignant melanoma (hereafter referred to as melanoma) is a cancer arising from melanocytes, the specialized pigment-producing cells residing in the epidermis of the skin. Although melanoma accounts for less than 5% of all the dermatological cancers, yet it is responsible for more than 75% of skin cancer-related deaths. Detection and subsequent surgical resection of melanomas at early-stage (radial growth phase) prevent malignant progression in most cases. However, patients with deep primary tumours or tumours metastasizing to the regional lymph nodes, frequently develop distant metastases. At this stage, melanoma becomes refractory to current therapies and has a very poor prognosis[1,2], with a median survival rate of 6-9 months and a 5-year survival rate of less than 5%. Therefore, the time of the diagnosis is crucial for the survival of patients. In addition, it is very concerning that, the overall incidence of melanoma continues to rise, with a worldwide annual increase estimated to be ~3-7% for Caucasians, in particular[1].

1.2. Melanoma: a combination of environmental and genetic factors

Melanoma, as it is emerging for several other cancer types, results from the interplay between environmental and genetic factors. A well-established risk factor in melanomagenesis is solar UV (**U**ltra**V**iolet) exposure. The magnitude of the risk depends on several variables, such as, the patterns (intermittent or cumulative UV) and the frequency (excessive or moderate) of sun exposure, as well as inherited susceptibility to its effects[1]. At the genetic level, three main cell-signalling pathways have been identified to play a crucial role in melanoma onset and development[1,2]:

1. Ras (**Ra**t **s**arcoma)/Raf (**R**apidly **a**ccelerated **f**ibrosarcoma)/MEK (**M**APK/**E**RK **K**inase)/ERK (**E**xtracellular-signal-**R**egulated **K**inase) signalling. The most common mutated molecule within this pathway is BRAF (one of the three human *Raf* genes, together with *ARAF* and *CRAF*), which is

mutated in 50% to 70% of all melanomas. The most common mutation occurs at the level of the codon 600 and results in the substitution of a glutamic acid with a valine within the protein sequence of BRAF (i.e. V600E), causing constitutive activation of the ERK signalling, which promotes tumour growth. Another common mechanism causing hyperactivation of this pathway is through gain-of-function mutations in *NRAS* (one of the three *Ras* genes in humans), which is mutated in 15%-30% of all melanomas. Also ERK has been found to be activated by autocrine production of growth factors and is hyper-activated in nearly 90% of human melanomas[2].

2. The phosphoinositide-3-OH kinase [PI(3)K]-Akt/PTEN (**P**hosphatase and **TEN**sin homolog) pathway: this pathway, which is hyper-activated in a high proportion of melanomas[2] due to activating mutations in *Akt*, or loss of the tumour suppressor gene *PTEN*, is crucially involved in sustaining proliferation, growth (increase in cell mass) and motility[2].

3. MITF (**MI**crophthalmia-associated **T**ranscription **F**actor): is a transcription factor belonging to the MiT/TFE transcription factor family that includes also TFEB (**T**ranscription **F**actor **EB**), TFE3 (**T**ranscription **F**actor **E3**) and TFEC (**T**ranscription **F**actor **EC**), and is a master regulator of melanocyte biology. This is in part because it regulates the expression of melanogenic proteins such as tyrosinase, silver homologue gp100 (**g**lyco**p**rotein100) and MART-1 (**M**elanoma-associated **A**ntigen **R**ecognized by **T** cells-**1**), also known as Melan-A, as well as melanoblast survival and melanocyte lineage commitment. MITF is expressed in most human melanomas and its target genes are frequently used as diagnostic markers. Constitutive expression of MITF is essential for melanoma cell proliferation and survival[2]. Because of the relevant role MITF plays in melanomagenesis, it has been suggested to act as lineage-specific oncogene[3].

Apart from these three major signalling pathways, several other genetic alterations in crucial regulators of apoptosis contribute to the notorious apoptosis-resistant

phenotype of advanced melanoma. These include but are not limited to: the overexpression of *Bcl-2 (***B**-**c**ell **l**eukaemia/**l**ymphoma-**2**); the loss or mutation of p53 or the silencing of APAF-1 (**A**poptotic **P**eptidase **A**ctivating **F**actor-**1**); or the constitutive activation of key pro-inflammatory and pro-survival pathways including *NF-κB* (**N**uclear **F**actor ***k***appa **B**eta).

Additional pro-melanoma signals may also come from the upregulation of the β-catenin pathway via loss or silencing of adenomatous polyposis coli (*APC*), or from rare activating mutations in β-catenin (*CTNNB1*). Also oncogenic drivers dysregulating the cell cycle (*CDKN2A/CDK4/CCND1*), are known to fuel melanoma and the complex genetic and mutational landscape of melanoma is further modulated by several epigenetic mechanisms and through various classes of non-coding RNAs[4].

1.3. Melanoma: therapeutic options

Melanoma aggressiveness and lethality have incited constant efforts in the development of novel and more efficacious therapies. In spite of this, the current available therapeutic approaches against metastatic melanoma are still inadequate or show success only in a limited subset of patients. This is largely caused by the activation of various melanoma cell-autonomous or cell non- autonomous resistance mechanisms. The main therapeutic options against melanoma include (apart from surgical excision): radiotherapy, chemotherapy, targeted therapies and immunotherapy[1]. Below there is a brief description of these approaches:

Radiotherapy

Radiotherapy is a local treatment administered to melanoma patients subjected to dissection of tumour and lymph nodes. Postoperative radiotherapy is advised to patients with inadequate resection margins, since it can improve local tumour control and help limiting the spreading of the tumour. This therapeutic intervention is also used as palliative therapy for patients at the terminal stage of the disease[5].

Chemotherapy

The use of chemotherapeutics for the treatment of melanoma still occurs, despite the well-known resistance of this cancer to them, as well as the limited improvements these drugs bring to the long-term survival of the patients[6]. The agents used for systemic treatment against melanoma include microtubule-targeting toxins and DNA-alkylating agents. Within the latter category, DTIC (Dacarbazine) – which for decades has served as a therapeutic standard-of-care in patients with inoperable metastatic melanoma - has received approval by the FDA (**F**ood and **D**rug **A**dministration). Given the modest effects provided by single agent therapy, combination treatments have also been tested. However these approaches fail to increase patient's survival and are also associated with considerable toxicity[7].

Targeted therapy

As previously mentioned, the Ras-Raf-MEK-ERK pathway plays a crucial role for melanoma onset and development. Therefore, not surprisingly, tremendous efforts have been put into the development of drugs able to efficiently block the constitutive activation of this pathway. These attempts have been payed off in 2011, when the FDA approved Vemurafenib (also named PLX4032, RG7204, RO5185426 or Zelboraf®) for the treatment of patients with unresectable tumours or metastatic melanoma harbouring $BRAF^{V600E}$ mutation[8]. By blocking this constitutively active form of BRAF, the MAPK pathway is inhibited and consequently its downstream signals stimulating survival, uncontrolled division and invasion[9]. Unfortunately, patients treated with Vemurafenib have showed only initial benefits and responses to the therapy. Indeed an early positive response[10] is generally followed by massive tumour growth (development of more aggressive clones) and hence therapy-resistance[11].

To overcome this $BRAF^{V600E}$ resistance, inhibitors against MEK have also been tried. In particular, patients harbouring unresectable and metastatic melanoma expressing the $BRAF^{V600E}$ or $BRAF^{V600K}$ mutations, when treated concurrently with $BRAF^{V600E}$ and MEK inhibitors (e.g. Trametinib), show durable clinical effects and lower toxicity

compared to treatment with the $BRAF^{V600E}$ inhibitor alone[12]. This combinatorial approach has received approval by the FDA in 2014 (https://www.fda.gov/).

Immunotherapy

Immunotherapy has been a successful therapeutic approach in the treatment of melanoma. Treatment with high dose IL-2 (**I**nter**L**eukin-2), which has been approved by the FDA, has been showed to be an effective anti-melanoma immunotherapy[7]. Although this treatment shows durable benefits only in a subset of patients, it proves that even a non-specific stimulation of the innate immune system and proliferation of T-cells[7] , as it is caused by IL-2, can be highly efficacious.

Recently, the FDA has approved a new approach for melanoma immunotherapy i.e. blockade of immune-checkpoints, supporting self-tolerance, with monoclonal antibodies directed against CTLA-4 (**C**ytotoxic **T**-**L**ymphocyte-**A**ssociated antigen **4**; Ipilimumab), PD-1 (**P**rogrammed cell **D**eath protein-**1**; Pembrolizumab) or the latter's' ligand. Other efficacious immunotherapy options being intensively tested in clinical trials include: neoantigens and/or DCs (**D**endritic **C**ell**s**)-based anti-cancer vaccines; and adoptive T cell transfer[13].

1.4. The role of the tumour microenvironment in melanoma

Accumulating studies indicate that the frequent failure of various anti-melanoma therapies is due not only to the intricate network of oncogenic pathways driving melanomagenesis, but also to the complex pro-tumourigenic and immunosuppressive TME (**T**umour **M**icro**E**nvironment) established by this tumour. The TME strongly contributes to melanoma's notorious heterogeneity and plasticity. The interchange of signals between cancer cells and noncancerous cells resident within the TME (i.e. stromal cells) has the ability to significantly influence tumour biology and progression[4,14]. Melanoma cells are able to educate stromal cells and in particular cells of the immune system in large part due to their enhanced ability to secrete pro-tumorigenic cytokines/chemokines and release exosomes[15]. The enhanced secretory capacity of melanoma cells is ensured and sustained by their ability to hijack various

homeostatic processes regulating proteostasis, such as the endo-lysosomal system, the ER (**E**ndoplasmic **R**eticulum) stress response and macroautophagy (henceforth referred to as autophagy)[15].

Human melanomas are relatively more immunogenic, as compared to certain other cancer types, owing to the presence of a diversity of tumour antigens (to such an extent that practically all the main principles of tumour immunology have been validated experimentally in this tumour system). However, in the majority of cases, the immune response to these antigens fails to counteract melanoma immune escape. This disparity is attributable to the ability of melanoma cells to evade the immune system via various processes, including (but not limited to) (i) subversion of various immunosurveillance mechanisms and (ii) blockade of the immune-mediated recognition of the cancerous cells. In particular, persistent melanoma antigen exposure, as well as loss of some highly immunogenic melanoma antigens can together blunt anti-melanoma immune response, where the former causes T cell exhaustion whereas the latter promotes escape from T cells directed towards those particular antigens. Nevertheless, both scenarios are typically accompanied by the melanoma-induced hyper-activation of immune response-inhibitory checkpoints that blunt the activity of cytotoxic T-cells. Pro-inflammatory and/or cytotoxic T cells are also further suppressed by the presence of immunosuppressive immune cells within the melanoma TME, namely TAMs (**T**umour **A**ssociated **M**acrophage**s**), T_{regs} (**T** **reg**ulatory cell**s**) and MDSCs (**M**yeloid-**D**erived **S**uppressor **C**ell**s**)[14]. Additionally, the inefficient killing of melanoma cells is also supported by the over-production of negative modulators of immune cells, as well as by the loss of both MHC-I and MHC-II (**M**ajor **H**istocompatibility **C**omplex class-**I** and **II**)[14]. Another factor frequently observed in solid tumours including melanoma that boosts their aggressiveness is hypoxia, i.e. a reduction in the normal tissue oxygen tension due to inadequate blood supply. In order to survive and further proliferate, cancerous cells respond to this hypoxic stress by undergoing genetic and adaptive changes that ultimately also confer them resistance to therapeutic treatments[16]. Interestingly, a recent research has showed that in melanoma cells, hypoxia downregulates MITF. In particular, decreased expression or deletion of MITF, is sufficient to increase melanoma metastatic potential[17]. It is arguable that, in spite of the

oncogenic activity of this transcription factor, inhibiting MITF-driven expression of melanoma antigens such as MART-1, TYRP-1 (**TY**rosinase-**R**elated **P**rotein **1)** or gp100, may favour escape from immunosurveillance mechanisms, thereby facilitating melanoma metastatic spreading[18,19].

As mentioned above, melanoma cells are able to exploit key housekeeping processes like autophagy to further their growth advantage. Autophagy is an evolutionarily conserved catabolic pathway, occurring at basal level in all cells and playing a key role in quality control, by promoting turnover of long-lived proteins and organelles, as well as degrading (defective or damaged) cellular components. Since autophagy supports homeostasis and survival and provides an alternative energy supply to fulfil the metabolic needs of a cell, it is not surprising that tumours exploit this process to support their progression, particularly advanced tumours which are exposed to a microenvironment with limited nutrients and oxygen availability[20]. It has been found that, in metastatic melanoma, essential autophagy genes (such as *ATG5*) not only support survival of melanoma cells within the nutrient-deprived TME , but have also additional cell-non autonomous effects on stromal cells, including immune cells, by means of surface-bound phagocytic signals on cancer cells or via the release of soluble chemotactic signals[21]. One pro-autophagic molecule in particular, which is drawing increasing attention in melanoma, is the focus of this thesis, i.e. BNIP3.

1.5. BNIP3: an ambiguous 'BH3-only' molecule

BNIP3 (**B**cl-2 [**B**-**c**ell **l**eukaemia/**l**ymphoma-**2**]/Adenovirus E1B] **N**ineteen kD **I**nteracting **P**rotein **3**) is a protein found both in the nucleus, as well as in the cytoplasm (in inactive state) such that, upon activation, it localises to the mitochondria to carry out its function. BNIP3 is a 'BH-3 only' protein belonging to the Bcl-2 family of cell death regulators. All members of this family possess at least one of the four shared Bcl-2 homology domains (BH1, BH2, BH3, BH4), also called conserved regions of homology. These domains are required to mediate the interactions among the members of this family and their different combinations determine if the protein will induce apoptosis or inhibit it[22]. This group of proteins, which include both pro-

apoptotic (e.g. multidomains Bax [**B**cl-2-**a**ssociated **x** protein] and Bak [**B**cl-2-**a**ntagonist/**k**iller] and various BH3-only proteins) and anti-apoptotic (e.g. Bcl-2 and Bcl-X_L [**B**-**c**ell **l**ymphoma e**x**tra-**l**arge]) ones, plays critical roles in this type of cell death. However, some members of the Bcl-2 family are also involved in non-apoptotic processes, such as autophagy. In line with this, despite being a pro-death molecule, BNIP3 has also a pro-survival role, playing hence a dual role depending on the context[23].

In the following sections, a deepened description of BNIP3 structure and functioning, as well as of its role in both physiological and pathological conditions - including cancer- with particular attention to melanoma, is provided.

BNIP3: molecular structure

BNIP3 is a protein (M_w=21.5 kDa) composed of 194 amino acids and five major domains, which consist from the N- to the C-terminus[21,22,24,25], (***Figure 1***) of*:*

1. **PEST domain** (**P**: proline, **E**: glutamic acid, **S**: serine, **T**: threonine and **D**: aspartic acid) which target BNIP3 for degradation. Particularly, this sequence is flanked by amino acids residues (i.e. histidine, arginine/lysine) usually associated with proteins subjected to turnover by proteasome-mediated degradation. Towards the N-terminus there is a conserved cysteine residue (Cys64) involved in the formation of a disulfide bond in the homodimer, hence conferring stability to the BNIP3 dimer;

2. **LIR** (**L**C3 [Microtubule-associated protein 1A/1B-**L**ight **C**hain **3]**-**I**nteracting **R**egion**):** a N-terminal tetrapeptide, WVEL motif[26], found within serine-rich PEST domain associated with protein degradation, allows BNIP3 to interact with LC3 and the autophagy machinery. This region confers to BNIP3 the property of an autophagy receptor;

3. **BH3 domain** (**B**cl-2 **H**omologous): which is the single homology domain shared with other Bcl-2 members. The BH3 domain of BNIP3 has a weaker affinity for

pro-survival proteins compared to other pro-apoptotic BH3-only proteins. This aspect might be linked to the reduced pro-apoptotic activity observed for BNIP3, as compared to other BH3-only proteins;

4. **CD** (**C**onserved **D**omain) domain: which contains 16 amino acids residues and which is immediately adjacent to the BH3 domain;
5. **TM** (**T**rans **M**embrane) domain: which is found at the Carboxyl-terminus and anchors BNIP3 to the mitochondrial outer membrane. The TM domain is crucial for triggering apoptosis, since its deletion abolished the pro-death activity of BNIP3 (mutants with defects in this domain are unable to localize in the mitochondria). Furthermore, BNIP3 interaction with the anti-apoptotic Bcl-2 family members occurs via this C-terminal domain, rather than via the usual BH3 domain, making BNIP3 an atypical member of its family. The TM domain governs not only BNIP3-mitochondrial localization, but also its homodimerization and function. When inactive, BNIP3 is loosely associated with the mitochondria, conversely when active it is integrated as a homodimer. However, other studies have showed that dimerization of BNIP3 might be not critical for cell death induction[24].

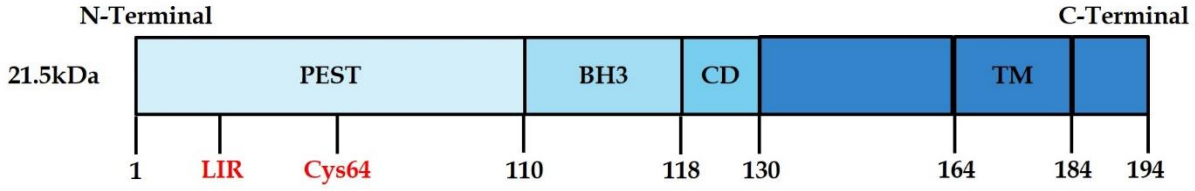

Figure 1: Molecular structure of BNIP3.
Schematic representation of the domain organisation of BNIP3, showing its different domains. The reader is referred to the text for the in-depth dissertation. (*Figure* adapted from Vasagiri *et al.*, 2014)[24].

BNIP3: expression, mechanisms of functioning and regulation

BNIP3 is not a ubiquitous pro-apoptotic protein. Under physiological conditions, it is expressed at low level in skeletal muscles and brain. BNIP3 expression is inducible

in both normal and cancerous tissues that undergo hypoxia or hypoxia-like conditions. Other stimuli, such as nitric oxide or arsenic trioxide, are also reported to induce BNIP3 expression[22]. Being involved in the modulation of cell death and survival, BNIP3 expression needs to be strictly regulated, a pre-requisite to maintain mitochondrial integrity and hence cell survival[27]. BNIP3 promoter contains two HRE sites (HRE1: 5'-CACGTC-3' located at -206 base pair and HRE2: 5'-CACGTG-3' located at -609 base pair). The HRE2 is required for hypoxia-induced activation of BNIP3. Hence BNIP3 is a direct transcriptional target of HIF-1α (**H**ypoxia **I**nducible **F**actor-**1α**)[23,28]. Indeed BNIP3 is a hypoxia responsive gene whose expression is triggered under conditions of low oxygen concentration or, when HIF-1α is overexpressed[21,22,24,25]. Once stabilised (under hypoxia), HIF-1α forms a HIF-1 heterodimer, together with HIF-1β, which is subsequently able to bind the HRE of the target gene. However, since the HIF-2α (**H**ypoxia **I**nducible **F**actor-**2α)** isoform negatively regulates BNIP3 expression[23], the relative expression levels of HIF-1α and HIF-2α determine the extent to which BNIP3 is induced, an aspect particularly relevant in hypoxic tumours[23]. Additionally, the transcription of BNIP3 is also modulated by the NF-*κB* signalling pathway, since the NF-*κB* response elements are located within the BNIP3 promoter[22].

Apart from HIF-1α, other transcription factors have also been identified to contribute to BNIP3 expression under non-hypoxic conditions. The evidence for this comes from studies showing that BNIP3 is over-expressed even in some well-vascularized tumours and non-hypoxic cells[22]. Several non-hypoxic factors, causing BNIP3 expression, have been identified. Specifically, the zinc-finger PLAGL-2 (**PL**eomorphic **A**denomas **G**ene **L**ike-**2**), which is expressed not only in response to hypoxia, but also during iron deficiency, might act independently or synergistically with HIF-1α to activate BNIP3 promoter during hypoxia[23, 25]. The precise site of interaction of PLAGL-2 is still unknown but several GC-rich boxes of BNIP3 promoter might be potential candidates[25]. Also, there are studies suggesting that under normal conditions, BNIP3 expression is regulated by post-translational modifications, as well as epigenetic regulation[24]. This latter occurs via DNA methylation of BNIP3 promoter (located in one of its CpG islands) in the region of the transcription start site[24]. Epigenetic regulation of BNIP3 has been implicated in the pathophysiology of various diseases[24],

thus unravelling the complex interplay of various genetic and epigenetic mechanisms contributing to BNIP3 expression[24].

Concerning BNIP3 role, numerous studies have highlighted that this atypical BH3-only protein can contextually play a pro-death or pro-survival role, the latter fulfilled via autophagy mostly, but not exclusively, in an hypoxia-induced manner[24,29].

There are three primary mechanisms for cell death induced by BNIP3: apoptosis, necrosis and autophagic cell death (***Figure 2; see page 28***). Although BNIP3 belongs to Bcl-2 family and, as described above, owns a BH3 domain, it localizes to the mitochondrial membrane and has a pro-apoptotic activity, its mitochondrial membrane permeabilization function differs from that of other BH3-only proteins. However like other members, also for BNIP3, activation of Bax/Bak is essential to mediate the mitochondrial membrane permeabilization and to induce Cytochrome *C* release[24]. Nevertheless it is unclear how BNIP3 activates Bax and Bak although it is likely that, as the other BH3-only proteins, it indirectly releases these two molecules by sequestering Bcl-2 and Bcl-X_L[23]. Additionally, other studies have showed that, BNIP3 (when overexpressed) mediates mitochondrial dysfunction also by opening of the mPTP (**m**itochondrial **P**ermeability **T**ransition **P**ore)[24]. This latter event abolishes the electrochemical gradient of the mitochondria electron transfer chain, causing mitochondrial swelling, ROS (**R**eactive **O**xygen **S**pecies) production and bioenergetics failure, a condition precipitating necrotic cell death[22]. Hence, BNIP3 has the ability to activate necrotic cell death (via mPTP opening) or apoptotic cell death (through Bax/Bak), in a context-dependent manner[23]. This BNIP3-regulated, mPTP opening-driven necrosis is intriguing since this is recognised as a programmed form of necrosis. Indeed it has recently emerged that necrosis is not an exclusively accidental phenomena but can also be orchestrated in a molecularly programmed fashion via several subroutines including (but not limited to) necroptosis, mitochondrial permeability transition-driven necrosis and ferroptosis[30]. Unfortunately, the link between BNIP3 and necroptosis or ferroptosis is not completely clear yet. However, a study reported that BNIP3 can be transcriptionally regulated by TNF and contributes to a necrostatin 1 inhibitable cell death pathway elicited by this cytokine in A549 lung adenocarcinoma cells[31]. It was shown that TNF stimulates the insertion of BNIP3 into

mitochondria, causing an increased production of ROS. Although the latter study lacks genetic validation of the induction of necroptosis by TNF and mechanistic evidence linking TNF cell death signalling to BNIP3, it suggests that under certain conditions or in certain cell types, the ability of mitochondria-associated BNIP3 to generate excessive production of ROS may contribute to necroptosis execution.

Furthermore, several studies have recently revealed a connection between autophagy (even specifically mitophagy) and regulation of necrosome with crucial implications for the mechanics and kinetics of necroptosis[32,33]. To this end, considering BNIP3's role in mitophagy regulation (***see page 29***) it can be speculated that a systematic study may well be able to find at least an indirect (if not, direct) role for BNIP3 in necroptosis regulation.

Consequently, several emerging studies highlight the ability of BNIP3 to regulate autophagy, with outcomes that –depending on the type of stress or cellular context- may range from cell survival to (autophagic) cell death. At the molecular level, BNIP3 may compete with Beclin-1 for the binding to Bcl-2 or Bcl-X_L complexes and the resulting displacement may induce autophagic cell death[26,34,35].

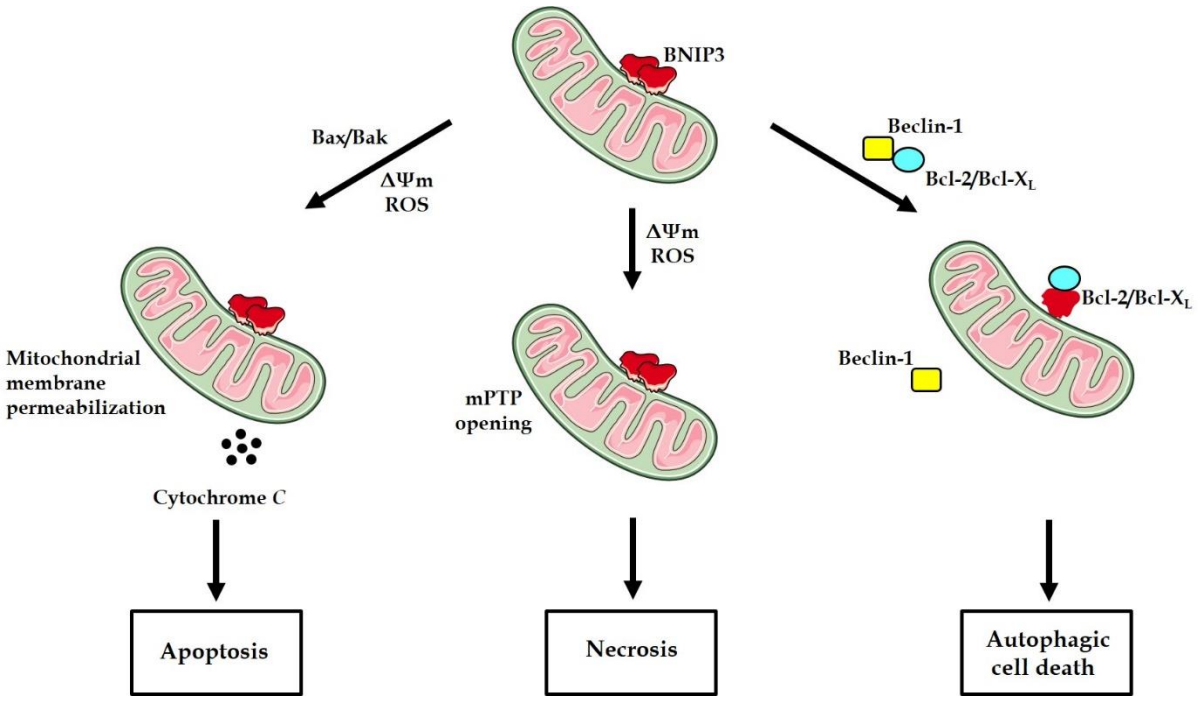

Figure 2: Mechanisms of functioning of BNIP3.
Schematic representation of the different pathways induced by BNIP3 activation, ranging from cell survival to cell death (through caspase-mediated apoptosis or necrosis). The reader is referred to the text for the in-depth dissertation. (*Figure* adapted from Chinnadurai *et al.*, 2009[25]; illustrations taken from https://smart.servier.com/).

Apart from the above-mentioned mechanisms, it has also been suggested that BNIP3 is unable to elicit cell death independently of other regulators and/or co-factors such as hypoxia-associated stressors (e.g. glucose deprivation, growth factor deprivation, intracellular acidosis)[23]. Alternatively, it is also possible that BNIP3-mediated cell death will rely on other regulators, in a cell-type dependent fashion. This hypothesis is supported by the findings that the cell surface molecule CD47 (**C**luster of **D**ifferentiation **47**) (a more detailed description of which, will be given on pages 32-35) has been found to be an interacting partner of BNIP3[36]. This latter binding involves BNIP3's TM domain, and the membrane-spanning domain of CD47. Under stress conditions, this interaction induces death of T-cells, via a process that does not imply BNIP3's transcriptional upregulation[23,24].

However, accumulating evidence indicate that BNIP3 has also pro-survival functions that are exerted mainly through its ability to regulate mitochondria clearance (mitophagy) and dynamics. Under hypoxic conditions, mitochondria-associated BNIP3 through its LIR domain is able to target the damaged mitochondria to the autophagy machinery (via mitophagy), preventing in this way ROS production and Cytochrome *C* release, events that otherwise would trigger caspase activation and apoptosis[37]. This suggests that, under hypoxic stress, BNIP3-induced autophagy might serve as a mechanism to rescue the cell from the adverse effects of BNIP3-induced mitochondrial dysfunction until a critical point of no-return is reached[23].

In cardiac myocytes, mitochondrial autophagy in response to BNIP3, involves mitochondrial fission mediated by Drp-1 (**D**ynamin-**r**elated **p**rotein-**1**) and recruitment of the E3 ubiquitin ligase Parkin to the mitochondria[38]. Furthermore BNIP3 interacts with the serine/threonine kinase PINK-1 (**P**TEN-**IN**duced putative **K**inase-**1**) to facilitate Parkin recruitment and PINK-1/Parkin-mediated mitophagy[39]. However BNIP3 can also clear dysfunctional mitochondria through Parkin/PINK1 independent mechanisms[40] and via a newly described endosomal pathway[41,42] governed by the small GTPases family member Rab5. Finally, BNIP3 has been showed to limit mitochondrial mass and maintain mitochondrial integrity in the liver (ultimately affecting lipid metabolism)[43].

BNIP3: a "Giano bifronte" in pathological conditions

Being both a pro-death as well as a pro-survival molecule, BNIP3 represents a double-edged sword protein which, not surprisingly, has been implicated in several pathophysiological functions. In particular, BNIP3 has been found to partake in the pathology of heart failure, since it regulates mitochondrial turnover and, mitochondrial density is really high in adult cardiac myocytes[24]. Several studies have implicated hypoxia-induced BNIP3 in cardiac myocyte-death (via autophagy or apoptosis) during myocardial infarction[22], suggesting that BNIP3 may be a potential therapeutic target for this pathology[24].

Apart from being involved in cardiovascular disorders, BNIP3 also plays a key role during synovial hyperplasia, rheumatoid arthritis[24], neuronal cell death after stroke as well as in neurological disorders, such as Alzheimer's disease[24].

BNIP3 duality has been observed also in cancer, where it can act as both tumour-enhancer and tumour-suppressor, in a context-dependent manner and mainly under hypoxic conditions. Indeed, BNIP3 plays an important role not only in (hypoxia-) induced death of normal cells but also of the malignant ones and its expression is considerably high in the poorly oxygenated areas, experiencing hypoxic stress, of some solid tumours[22].

The ability of hypoxia to select populations of cells with marked resistance to apoptosis, takes place via upregulation of pro-survival factors and/or down-regulation of pro-death molecules. Among the mechanisms by which a cancer cell can escape hypoxia-induced apoptosis, there is the epigenetic silencing of pro-death genes, which ultimately confer a survival advantage[23]. Hence, the ability of tumour cells to elude apoptosis in the hostile microenvironment of solid tumours is a crucial factor for tumour progression and for the development of resistance to chemotherapy[23]. In this context, hypoxia-driven modulation of BNIP3 expression might represent an important step for the development of a metastatic phenotype. However, one mechanism through which BNIP3 may instead repress tumour growth under hypoxia relies on its ability to bind Rheb, an activator of mTORC1 (the **m**echanistic **T**arget **O**f **R**apamycin **C**omplex **1**) pathway, thereby blocking its activating ability. Suppression of mTORC1 by BNIP3 may explain the inhibitory effects of hypoxia on mTOR signalling and the mTORC1-mediated growth advantage provided by the BNIP3 knockdown in certain tumour models [44].

BNIP3 expression has been showed to be heightened in peri-necrotic areas commonly found in solid tumours, concomitant with upregulation of HIF-1[22]. Also, BNIP3 has been found to co-localize with HIF-1α expression[23]. Hypoxic stress within the interior tumour mass favours cancer cell's invasive potential and pro-angiogenic signalling. In line with this, BNIP3 expression has been found to be associated with increased persistence of growth factors like VEGF (**V**ascular **E**ndothelial **G**rowth **F**actor), bFGF (**b**asic **F**ibroblast **G**rowth **F**actor) and PDGF (**P**latelet **D**erived **G**rowth **F**actor)[24]. Thus

it seems that, there is a balance between death and survival signals induced by hypoxia, that need to be considered when analysing the effect of BNIP3 on cancer prognosis and progression[22].

Although BNIP3 tends to be overexpressed in the hypoxic regions of the tumour, its pro-apoptotic activity[22] can be prevented by sequestering BNIP3 into the nucleus. The mechanism that allows BNIP3 to localize in the nucleus is unknown as well as its function in this cellular compartment.

Several cancers (e.g. breast, lung and cervix) express high levels of BNIP3 and various studies have highlighted the relevance of BNIP3 in cancer progression and resistance to therapy. The silencing and subsequent methylation of BNIP3 gene occur in a significant number of cancers, particularly in pancreatic cancer (where silencing of the CpG islands of BNIP3 promoter by methylation is crucial for pancreatic cancer survival under hypoxia), but also in colorectal, gastric and haematopoietic cancers[22]. However BNIP3 silencing is tissue specific during tumour development or progression and despite the fact that this methylation influences tumour treatment and prognosis, the exact mechanism is still not clear[24].

Although the majority of the studies have disclosed a pro-tumour effect of BNIP3, a recent work by Chourasia *et al.*, has revealed, for the first time, that BNIP3 can behave also as tumour suppressor in a clinically relevant mouse model of mammary tumorigenesis, proving once more the dual nature of this ambiguous molecule[45].

Thus, more comprehensive *in vitro* and *in vivo* studies are necessary to fully understand BNIP3' function and role in cancer and cancer therapy.

BNIP3: a crucial player in melanoma?

The role of BNIP3 in melanoma cells has been partly disclosed[29]. Specifically, BNIP3 is expressed at basal level in melanoma cells in normoxia and is further stimulated by hypoxia, in order to assist in critical pro-melanoma functions. Among them, noteworthy are BNIP3's ability to promote migration, survival and long-term clonogenic growth of melanoma cell lines together with its crucial role in determining the actin-based cytoskeletal architecture and hence cellular morphology. BNIP3 is also crucial for mitochondrial clearance and viability[29]. Moreover, melanoma cell-

associated BNIP3 is important to prevent degradation of CD47, a key "don't eat me" signal enabling cancer cells to evade the anti-tumour immunity by suppressing the phagocytic functions of the innate immune cells[29]. Remarkably, there is a strong correlation between the transcript levels of *BNIP3* and *CD47* in melanoma patient samples and both genes tend to act as negative prognostic factors in these patients[29]. This suggests that, in melanoma cells, BNIP3 supports cell-autonomous properties fuelling *in vitro* growth, with a putative clinical link.

1.6. CD47: a BNIP3-partner

As previously mentioned, an interesting interactor of BNIP3 is CD47. In the following sections, a more detailed description of its structure and functioning in both physiological and pathological conditions is provided.

CD47: molecular structure and mechanism of functioning

CD47, also known as IAP (**I**ntegrin **A**ssociated **P**rotein), is a 50 kDa protein belonging to the Ig (**I**mmuno**G**lobulin) superfamily and it is expressed on almost all cells. Its structure consists of five transmembrane regions together with a single Ig-like domain and a short alternatively spliced cytoplasmic tail. When CD47 binds - via its Ig-like domain - the N-terminal domain of the myeloid inhibitory immunoreceptor SIRPα (**SI**gnal **R**egulatory **P**rotein **α**) on phagocytic cells, an immune inhibitory signal propagates within these latter cells (***Figure 3***). This signal ultimately inhibits host cell phagocytosis, therefore depicting CD47 as a 'don't eat-me' signal. Mechanistically, CD47 induces the phosphorylation of ITIMs (**I**mmunoreceptor **T**yrosine-based **I**nhibitory **M**otifs) on SIRPα allowing the subsequent recruitment of two kinases, SHP-1 and SHP-2 (**S**rc **H**omology **P**hosphatases-**1** and -**2**). However only SHP-1 is associated with the inhibitory function of SIRPα in macrophages[46], since SHP-1 mediates dephosphorylation of non-muscle myosin IIA at the phagocytic synapse as a result of the interaction between macrophages-SIRPα and CD47 on the host cell, ultimately limiting phagocytosis[47]. On the other hand SHP-2 associated with SIRPα leads to phosphatase-dependent enhancement of the signal in several contexts[48].

Moreover CD47 engagement may also involve both heterotrimeric Gi protein-dependent and independent pathways, as well as signalling could occur via the integrin cytoplasmic tail[40,49].

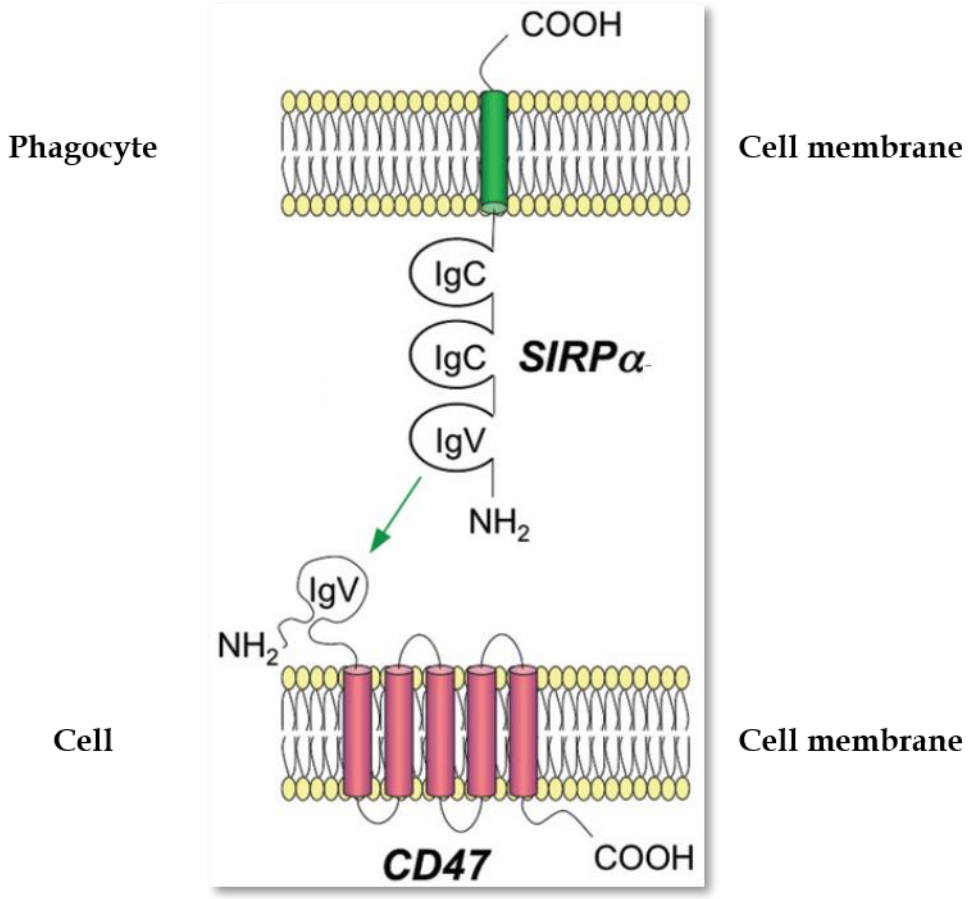

Figure 3: Molecular structure of CD47 and its interactor SIRPa.
Schematic representation of the molecular structure of CD47, showing its different domains and those of one of its interactor, SIRPα. The reader is referred to the text for the in-depth dissertation. (*Figure* adapted from Sick *et al.*, 2014)[50].

Although CD47-SIRPα interaction governs the broadest spectrum of intercellular functions, yet CD47 has also other interactors, namely (i) a narrow number of Integrins (*cis* interactions) and (ii) Thrombospondin (TSP), each of which induce downstream activation of specialized pathways. These latter range from (i) modulation of IgG-mediated phagocytosis, regulation of neutrophil inflammatory responses as well as

their adhesion, migration, phagocytosis and respiratory burst[51,52,53] to (ii) cell motility, proliferation and differentiation[54].

CD47 is also involved in the phagocytic clearance of apoptotic cells and several hypotheses have been formulated to explain this mechanism of action. The most obvious one is to assume a down-regulation of CD47 on the apoptotic cells and a concomitant up-regulation of pro-phagocytic ligands, as found on apoptotic neutrophils and fibroblasts[55]. Another possibility could be the segregation of CD47 away from phagocytosis ligands, since CD47 often redistributes into patches on apoptotic cell surfaces. These patches are areas of the plasma membrane different from those harbouring clusters of ligands for pro-phagocytic receptors[55,56].

CD47: a player in cancer

Being CD47 a 'don't eat-me' signal, as well as an important determinant of host cell clearance and/or killing by macrophages, the role played by CD47-SIRPα-interaction in cancer is intriguing. Specifically, CD47 has been found abundantly over-expressed on a variety of both solid tumours and hematopoietic malignancies, with the highest levels generally found on cancer stem cells[57]. Additionally, CD47, by inhibiting phagocytosis of cancer cells by macrophages, represents ultimately a cancer strategy to escape immunosurveillance by the innate immune system. In line with this, cancer cell-associated CD47 expression level represents an adverse prognostic factor, with patients showing relatively high CD47 expression levels having worse prognosis compared to those with lower CD47 expression levels[57,58]. Interestingly, CD47 is transcriptionally regulated by HIF-1α in breast cancer. In particular, HIF-1α regulates the expression of target genes that correlate with CD47 expression and this ultimately correlates with poor survival of these breast cancer patients[59].

Despite SIRPα signalling itself is not important for cancer metastasis and outgrowth, CD47-SIRPα interaction and subsequent SIRPα signalling do restrict the efficacy of cancer therapeutic antibodies[60]. In light of this and considering that tumours are to a certain degree immunogenic, CD47 can be used as target in cancer therapy, exploiting both innate and adaptive immune response, since not only macrophages, but also DCs and T cells, play a role at the interface with CD47. In particular, anti-CD47-induced

anti-tumour immune response and its therapeutic effects require $CD8^+$ T cells. Also the induction of Type I and II IFNs (**I**nter**F**ero**Ns**), as well as the responsiveness of DCs to Type I IFN and the increased DCs cross-priming of cytotoxic T lymphocytes are crucial for the therapeutic effect of the anti-CD47 treatment[61].

1.7.Phagocytosis: a duel between 'eat-me' and 'don't eat-me' signals

As already mentioned, CD47 is involved in phagocytosis at the interface with innate immune cells, mainly with macrophages.

Metchnikoff laid the foundations for the theory of phagocytosis in 1882 in Messina, while "amusing" himself with some echinoderms. By introducing a rose thorn under the skin of a transparent starfish, he performed his 'Eureka experiment' that made him the Father of the phagocytosis theory[62] .

In unicellular organisms, phagocytosis represents an important process for their nutrition, while in multicellular organisms it is a more articulate mechanism performed by specialized cells, called phagocytes. Phagocytosis is indeed a complex process used by the cells to ingest and eliminate pathogens, as well as to remove apoptotic cells, maintaining in this way tissue homeostasis and controlling important aspects not only of the immune responses, but also of inflammation[63].

Phagocytes can also recognize and phagocytose living cancer cells under some very specific conditions. For instance live cancer cells, coated with anti-CD47 antibodies[64,65] or genetically deficient in CD47[66], are phagocytosed rapidly by the macrophages. In some (but not all) cases, such phagocytic clearance of live cells may also cause the target (eaten) cell to undergo 'phagoptosis' within the phagocyte[64,67]. Interestingly, live cancer cells deficient in CD47 are particularly susceptible to CALR-mediated (**CALR**eticulin) phagocytosis by macrophages. Notwithstanding a limited number of studies, it is clear that such phagocytosis of live cancer cells has major implications for tumour immunosurveillance[66]. However, more research on clearance of live or largely intact cancer cells is required to understand the molecular mechanisms and the contexts behind its occurrence.

In the following sections, a summary about the mechanism of functioning of this process and about its player is discussed.

Phagocytosis: effector cells and their receptors

Phagocytosis consists of recognition and ingestion of particles larger than 0.5 μm into the so-called phagosome, a vesicle deriving from the plasma membrane. Both professional and non-professional phagocytes carry out this process. To the first category belong monocytes, macrophages, neutrophils, DCs, osteoclasts, and eosinophils. Non-professional phagocytes include endothelial cells, epithelial cells (in the skin or intestine) and fibroblasts. These two groups of phagocytes differ in the type of particles they can phagocytose. Specifically, the non-professional phagocytes cannot internalise microorganisms although play a role in the elimination of the apoptotic bodies. On the other side, the professional cells are able to eliminate microorganisms as well as cellular debris and to present their antigens to the cells of the adaptive immune system. This specifically applies to DCs, the major APCs (**A**ntigen **P**resenting **C**ells)[63].

In order to ingest particles, a phagocyte needs first to recognize them. This is achievable thanks to a wide array of receptors that after the recognition of the target start a signalling cascade that ultimately primes phagocytosis. The receptors on the plasma membrane of phagocytes are divided into two groups[63] (***Table 1***) :

1. **Opsonic**: that recognize opsonins, soluble molecules deriving from the host, binding to foreign particles, and hence tagging them for ingestion. To this group belong: antibodies, complement, fibronectin, mannose-binding lectin, milk fat globulin (lactadherin), F_cR (**F_c** **R**eceptor) and the CR (**C**omplement **R**eceptor). These two latter are the most important and characterised opsonic receptors, with the F_cRs binding the constant portion (F_c) of IgG or IgA antibodies and the CRs (e.g. CR3) binding to iC3b (located on the particle after complement activation).

2. **Non-opsonic**: that recognize directly molecular groups on the surface of the target. Among them, there are: (i) lectin-like recognition receptor (e.g. CD169, CD33); (ii) C-type lectins (e.g. Dectin-2, Mincle, DNGR-1); (iii) scavenger receptors; (iv) Dectin-1 (a receptor for fungal β-glucan). Intriguingly TLRs (**T**oll-**L**ike **R**eceptor**s**), although cooperate with other non-opsonic receptors to promote ingestion and to detect foreign particles, do not operate as phagocytic receptors. Furthermore, TLR3, TLR4 and TLR5 receptors have been found to differentially regulate the phagocytosis of dying cells by macrophages[68,69].

   Finally, also other receptors (e.g. SR-A or CD36) can recognize both apoptotic and microbial polyanionic targets but their signalling mechanisms are less well known.

Type of phagocytosis	Receptors	Ligands
Opsonic phagocytosis	Fc receptor family (FcγRI, FcγRIIA and FcγRIIA	Antibody-opsonized targets
	Complement receptors (CR1, CR3 and CR4)	Complement-opsonized targets
	α5β1 integrin	Fibronectin
Non-opsonic phagocytosis	Dectin 1	β-glucan
	Macrophage receptor MARCO	Bacteria (undefined specific ligand)
	Scavenger receptor A	Bacteria (diverse charged molecules)
	αVβ5 integrin	Apoptotic cells
Triggered (non-specific) phagocytosis	Toll-like* receptors	Various, including lipopolysaccharides and lipopeptides

Table 1: Main receptors involved in the phagocytic process and their ligands.
The table shows the different receptors and their ligands involved in the three types of phagocytosis (opsonic, non-opsonic, non-specific). (Table adapted from Underhill *et al.*, 2012)[70].
* *Signalling through TLRs promotes inflammatory immune responses, cytokine production and cell activation. TLRs-triggered phagocytosis is strongly associated with bacterial phagocytosis wherein individual TLRs facilitate phagocytosis in a differential fashion such that TLR9 is the best elicitor of phagocytosis*[71]*. For apoptotic cells, the role of TLR-triggered phagocytosis is less well-characterized*[72,68,].

Phagocytes possess various types of receptors and their cooperation determines the efficiency of the recognition, which is an essential step to allow the process of phagocytosis.

Phagocytosis: a multistep process

Phagocytosis is a complex and elegant process, which occurs, in a multistep fashion (***Figure 4)***. This process is hallmarked by four main phases: (1) Particle recognition; (2) Particle internalization; (3) Phagosome formation; (4) Phagolysosome maturation. Following is a brief description of each of these phases:

1. **Particle recognition:** particle detection and recognition is the step defining, in the majority of cases, the start of the phagocytic process. Specialized receptors on the cell membrane of phagocytes, recognize ligands on the surface of foreign particles directly (by binding molecules not found in higher organisms) or indirectly (via opsonins);

2. **Particle internalization:** once the cooperating receptors sequentially recognize and interact with the target, a signalling cascade begins, in order to remodel both the lipids in the plasma membrane and the actin cytoskeleton, to allow the formation of pseudopods (extensions of the membrane itself) around the target. More specifically, a depression of the membrane called 'phagocytic cup', is formed at the point of contact. Subsequently the membrane envelops the target particle and within few minutes, its distal ends are moved close to generate a new phagosome. Furthermore, the mode of internalization is dictated by the type(s) of phagocytic receptor(s) involved, the size of the particle being internalized, as well as the type of cell death pathaway. In particular, concerning the latter point, it has been observed that apoptotic cells are largely internalized by receptor-mediated phagosomal uptake, while necrotic cells tend to be internalised via macropinocytosis (a fluid phase uptake of small molecules), implicating the formation of multiple ruffles directed towards the necrotic debris[73,74];

3. **Phagosome formation:** once the particle is "wrapped", it pinches out from the plasma membrane as a new vesicle;

4. **Phagolysosome maturation:** when the particle is internalized by the early phagosome, it can fuse with vesicles, coming from the ER and Golgi apparatus, to generate the intermediary phagosome. This latter forms via a dynamic process implying the fusion of endocytic vesicles, as well as the fission of the secretory ones, ultimately resulting in the remodelling of the membrane and progressive acidification of the phagosome. Therefore, the composition of the phagosome membrane changes as well as its contents, turning into a phagolysosome, a vesicle able to destroy the particle ingested. Finally, the phagolysosome fuses with lysosomes, hence changing its membrane and inner characteristics and ultimately going through the phagolysosomal maturation. This last step consists of serial fusion and fission interactions between the new phagosome and early endosomes, as well as late endosomes and lysosomes. Ultimately, the mature lysosome (phagolysosome) has a different membrane composition able to accommodate a greatly acidic and degradative environment[63].

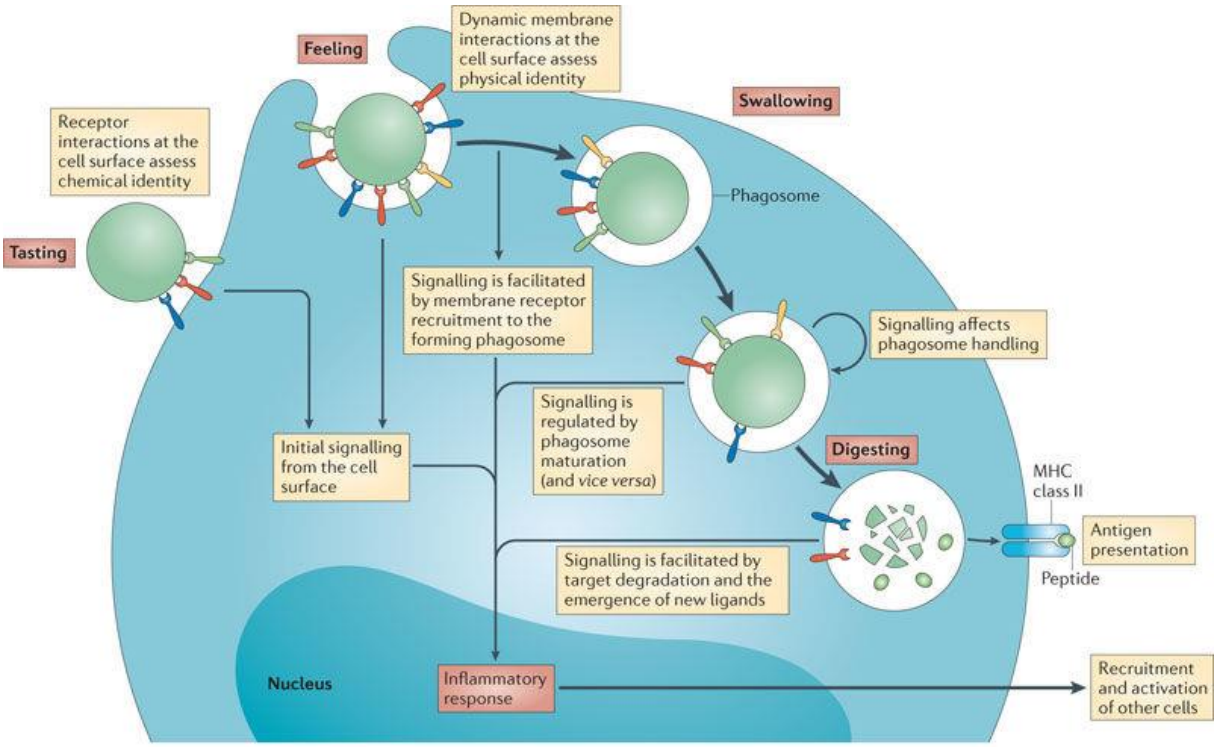

Figure 4: Major steps of the phagocytic process.
Schematic representation of the phagocytic process from the internalization of the target to the emission of downstream signals. The reader is referred to the text for the in-depth dissertation. (*Figure* taken from Underhill *et al.*, 2012)[70].

1.8.Macrophages: a spectrum of phenotypes and functions

Macrophages belong to the innate immune system and are one of the first lines of defence against pathogens, along with neutrophils. These cells, together with the mononuclear phagocytes, represent the cellular branch of the innate immunity and with DCs, have a crucial role in initiating, orientating and modulating several features of the adaptive immune response.

Macrophages are key players of the innate immune system found in all tissues and having a pivotal role in immunity and host defence, development, tissue repair and metabolic homeostasis. In particular, they provide protection by triggering inflammation, disclosing antimicrobial and tumoricidal features, regulating adaptive immune system activation, ultimately resolving the inflammation and promoting

tissue repair. They also contribute to the development of several organs and tissues (e.g. brain, bones, mammary gland) hence showing trophic functions[75].

Macrophages are typified by their extreme phenotypic and functional plasticity and versatility, as well as by their diversity. These features, even antagonistic, allow them to quickly adapt and efficiently respond to changes in the tissue microenvironment, by polarizing to specific functional phenotypes. However, these same characteristics make them detrimental in some contexts. The literature offers a wide array of works showing the role of macrophages in pathogenesis, going from cancer to immunodeficiency, from allergy to autoimmune diseases. Thus macrophages impact virtually all the known major pathologies, representing at the same time valuable therapeutic targets[75].

In physiological conditions, macrophages respond to their tissue microenvironment by modulating their morphological and functional aspects. In doing so, they may specialize into distinct resident populations: microglia (in the brain), alveolar macrophages (in the lungs), Kupffer cells (in the liver) or lamina propria macrophages (in the gut). In pathological conditions (microbial infection or sterile tissue damage) instead, they carry out host protective functions[75].

Macrophages-polarization

Plasticity is a key feature of the monocyte-macrophage lineage. The differential activation state of these cells is dictated, within the tissue microenvironment, by the integration of a set of signals, particularly tissue-specific signals, soluble and microbial factors. The results of this integration induce genetic re-programming, phenotypic changes and differential activation of macrophages, leading to the so-called "polarization". Although in a very simplistic way polarized macrophages are divided into two main categories, namely M1 ('classical') and M2 ('alternative'). However, between these two extremes there is a spectrum/continuum of phenotypes and functional states that further underline the complexity of macrophages-plasticity (***Figure 5***). Clearly, M1 and M2-macrophages receive different stimuli. Originally the nomenclature M1/M2 was used when referring to macrophage populations showing distinct nitrogen metabolism pathways (nitric oxide vs arginine production), after

stimulation with LPS (**L**ipo**P**oly**S**accharides) or IFN-γ (**I**nter**F**ero**N**-**γ**). The M1 and M2-macrophages classification mirrors the Th1 (**T** **h**elper type **1**) and Th2 (**T** **h**elper type **2**) paradigm. The classically activated macrophages are stimulated by Th1 cytokines (therefore are named M1), such as IFN-γ alone or together with bacterial products like LPS or inflammatory cytokines, e.g. GM-CSF (**G**ranulocyte **M**acrophage-**C**olony **S**timulating **F**actor) and TNF (**T**umour **N**ecrosis **F**actor) and have pro-inflammatory roles[75,76]. Upon stimulation, these macrophages release ROS and nitrogen intermediates, as well as secrete inflammatory (IL-1β [**I**nter**L**eukin-1β], TNF, IL-6 [**I**nter**L**eukin-6]) and immunostimulating cytokines (such as IL-12 [**I**nter**L**eukin-12] and IL-23 [**I**nter**L**eukin-23]) but low levels of the immunoregulatory cytokine IL-10 (**I**nter**L**eukin-10), ultimately eliciting the adaptive immune response. Hence M1-cells have cytotoxic effect and it can be easily inferred that they are involved in the battle against intracellular parasite, as well as against neoplastic cells. On the other hand, alternatively activated macrophages are differentiated via Th2 cytokines (therefore are named M2) such as, but not only, IL-4 (**I**nter**L**eukin-4) or IL-13 (**I**nter**L**eukin-13). The different forms of M2-macrophages usually show high levels of IL-10, variable ability to produce inflammatory cytokines and low levels of IL-12, IL-23 and IL-1β. They also show high expression of the scavenger, mannose and galactose-type receptors that explain their scavenging function as well as of Arg1 (**Arg**inase**1**), involved in the production of ornithine and polyamines. From a functional point of view, since they are involved in polarized Th2 response, M2-macrophages are engaged not only in parasite clearance, but also in the production of several growth factors that ultimately activate the process of tissue repair and remodelling, hence suppressing the adaptive immune response (immunoregulatory functions). This explains why, in cancer, M2-macrophages are prone to support tumorigenesis together with angiogenesis[76].

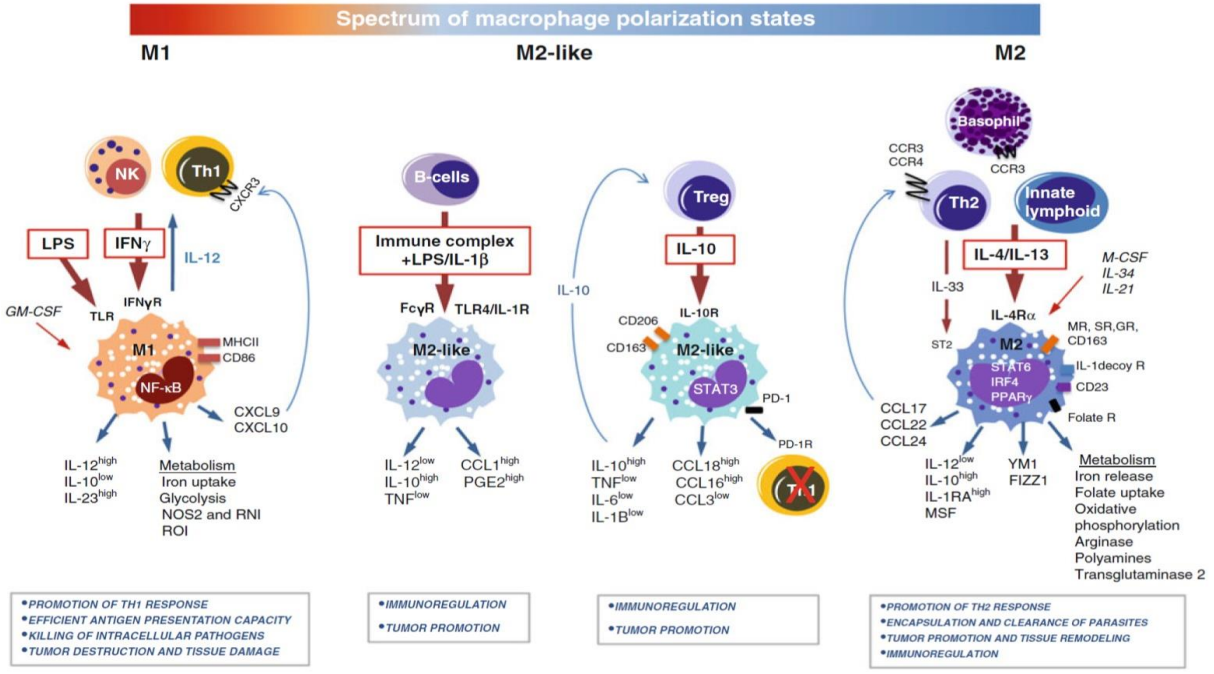

Figure 5: Macrophages-polarization.
Schematic representation of the polarized activation of macrophages. The figure depicts the three major polarization states: M1, M2 and M2-like and the associated inducing stimuli (red boxes above), functional properties (grey boxes below), key genes, metabolic features and transcription factors. Additional polarizing stimuli are indicated in *italics*. It is also reported the crosstalk between polarized macrophages and different subsets of lymphocytes. (*Figure* taken from Biswas and Mantovani, 2010)[75].

Despite the increasing interest in this field, the description of macrophages-activation is still confusing. However, Murray *et al.* recently published clarifying guidelines with the aim of proposing a common structure for the nomenclature of macrophage-activation and to which we address the readers for further in-depth readings[77].

As aforesaid, M1 and M2-polarized macrophages differ both in their production of chemokines and expression of the respective receptors. M1-cells release chemokines like CXCL-9 (chemokine [**C**-**X**-**C** motif] **L**igand-**9**) and CXCL-10 (chemokine [**C**-**X**-**C** motif] **L**igand-**10**) which are some of the most typical Th1 cell-attracting chemokines. M2-macrophages instead secrete the chemokines CCL-17 (**C**-**C** motif **L**igand-**17**), CCL-22 (**C**-**C** motif **L**igand-**22**) and CCL-24 (**C**-**C** motif **L**igand-**24**). Additionally, the polarization status of macrophages can be influenced (in limited contexts) by the

chemokines themselves, like CCL-2 (chemokine [**C**-**X**-**C** motif] **L**igand-**2**) promoting an M2-like phenotype or CXCL-4 (chemokine [**C**-**X**-**C** motif] **L**igand-4), inducing a unique phenotype characterised by a mixture of M1 and M2-features.

Another difference between these two subclasses concerns metabolism regulation, in particular glucose, folate and iron metabolism. For example, enzymes like Transglutaminase 2 is a M2-characteristic[75].

The functional M1/M2-polarization status has been observed also in pathological conditions (*in vivo* and *ex vivo*), ranging from parasite infections to cancer. Additionally, in these pathological conditions and their course, it is not rare to observe overlapping M1/M2-features, as well as shifts in the polarization status. Hence, despite the efforts made to differentiate the two phenotypes, it is unavoidable to have mixed populations of macrophages, a feature further proving the plasticity of this cell type.

Finally, it infers that subsets of polarized macrophages can be used as therapeutic targets[75].

1.9. Tumour Associated Macrophages (TAMs): active players in cancer

TAMs: origin

Similar to tissue macrophages, TAMs are extremely heterogeneous and able to respond to the surrounding environment, features that allow immune escape of cancer cells. TAMs derive from hematopoietic monocytic precursors circulating in the blood (or resident in the spleen) from where they are subsequently recruited into the tumour[75,78].

TAMs are not the only cells able to infiltrate solid tumours. They are accompanied by mast cells, MDSCs and neutrophils. In a clinically established tumour, cancer cells produce hematopoietic growth factors (like GM-CSF, IL-3) to stimulate the bone marrow myeloid progenitors and ultimately increasing the production of monocytes/macrophages. This allows the accumulation of TAMs, since the

proliferating potential of differentiated macrophages is very limited. However, macrophage-proliferation in peripheral tissues has also been observed to be determinant for their accumulation, particularly at sites of inflammation where M2-polarized macrophages are located. Chemotactic factors, mainly chemokines of the CC family (such as CCL-2), are involved in the recruitment of monocytes in the tumour, but other chemoattractants, i.e. GM-CSF and M-CSF (**M**acrophage-**C**olony **S**timulating **F**actor), VEGF and TGF-β (**T**ransforming **G**rowth **F**actor-**β**eta) of macrophages can also play a role. Once monocytes have reached the tumour, the majority of them may differentiate into mature macrophages if supported by the locally produced M-CSF (otherwise, they may stay-on as MDSCs). Several human tumours have been found to produce this cytokine and its production correlates with poor prognosis. Hence the local microenvironment drives the fate of monocytes towards a pro-tumour phenotype, also via factors like IL-6, IL-10, M-CSF, TGF-β and prostaglandins, like PGE-2 (**P**rosta**G**landin **E**-**2**). Given the extreme heterogeneity of TAMs, depending on the cancer type, disease stage and tumour location (normoxic vs hypoxic regions), different specific markers and transcriptional profiles may associate with different TAM subsets[75].

TAMs: pro-tumour functions

The pro-tumourigenic role of TAMs involve: (1) cancer cell proliferation and survival, (2) matrix remodelling, tumour invasion and metastases, (3) immunosuppression, (4) hypoxia and angiogenesis. It has been widely proven that TAMs and related myeloid cells, namely MDSC, as well as TEM (**T**IE-2 **E**xpressing **M**onocytes), have these functions. Following is a brief description of each of the effects of TAMs on tumour, largely based on the extensive book by Biswas & Mantovani (chapter 20)[75]:

1. **Cancer cell survival and proliferation:** several studies have demonstrated that macrophages-depleted animals have slower tumour progression and dissemination, a proof of the involvement of these cells in malignancies[79]. TAMs also support cancer stem cells or tumour initiating cells, a feature with an

obvious impact on tumour evolution and resistance to therapies. Interestingly, *in vivo* imaging studies have showed prolonged physical interaction between cancer cells and TAMs, with the latter extending cytoplasmic protrusions towards the former. Additionally, a direct effect on tumour proliferation is mediated via trophic and growth factors such as members of the EGF (**E**pidermal **G**rowth **F**actor), FGF and PDGF family. From their side, cancer cells are able to recruit and support macrophages-viability by realising M-CSF that in turn, produce EGF. TAMs also release active matrix-bound growth factors upon ECM (**E**xtra**C**ellular **M**atrix) degradation (a process actively mediated by these cells), as well as TNF and IL-6, cytokines with a major role in tumour growth. TNF in turn triggers the transcription factor NF-κB that thereafter activates a survival program in cancer cells. IL-6 instead, activates STAT-3 (**S**ignal **T**ransducer and **A**ctivator of **T**ranscription-**3**) pathway that as a result, induces the expression of genes involved in cell cycle progression, such as cyclin D and PCNA (**P**roliferating **C**ell **N**uclear **A**ntigen) and apoptosis suppression, like Bcl-X_L, Bcl-2, and Mcl-1 (induced **M**yeloid **L**eukaemia **C**ell differentiation protein). Finally, several factors released from cancer cells, such as cytokines, coagulation factors, enzymes and matrix proteins, can reach distant sites via the systemic circulation (either in soluble form, or bound to exosomes) preparing in this way the "soil" for pre-metastatic niche (as per the "seeding theory" of cancer dissemination). Subsequently macrophages and myeloid cells are recruited in these niches and actively collaborate to increase the survival of these disseminated cancer cells that otherwise would not be able to outlive the possibly hostile microenvironment at these new locations.

2. **Matrix remodelling, tumour invasion and metastasis:** resident stromal cells (endothelial cells, fibroblasts, leukocytes) and cellular matrix, exchange complex signals in normal tissues in order to keep tissue homeostasis and to repair tissues in case of injury. This exchange of signals is required to keep under strict control cellular processes like gene expression and differentiation, adhesion, growth, migration and death. On the contrary, tumour stroma has a

disrupted tissue architecture, characterised by activated fibroblasts and leukocytes, as well as irregular vessels. Likewise, the composition of some of its ECM proteins (e.g. alpha smooth muscle actin, fi bulin-1, fibronectin…) is different. TAMs also produce and secrete several of these ECM proteins (such as osteopontin, osteoactivin, various collagens, fibronectin) and interestingly, altered expression of ECM-related genes (by myeloid or other stromal cells), has been found associated with patient clinical outcome. Together with secretion of ECM proteins, TAMs also produce numerous proteolytic enzymes and actually, they can be considered the main cell type expressing protease activity in a tumour. The specific proteases that degrade matricellular proteins include members belonging to different families, such as MMPs (**M**atrix **M**etallo**P**roteases), cathepsins, hyaluronidases and ADAM (**A** **D**isintegrin **A**nd **M**etalloprotease) proteases, as well as heparinase, elastase, uPA (**u**rokinase-type **P**lasminogen **A**ctivator) and plasmin. The proteolytic degradation carried out by these proteases affects the stroma stiffness and removes the physical barrier between the cells, hence favouring the invasion of migrating cells, both neoplastic and endothelial cells. In fact, the disruption of integrin-mediated anchorage and FAK (**F**ocal **A**dhesion **K**inase) is crucial for tumour cells invasion into the nearby spaces.

Interestingly it has been showed that IL-4-stimulated TAMs has a cathepsin protease activity promoting tumour dissemination[80]. IL-4 is produced by CD4 T-cells infiltrating the tumour and it is relevant for the polarization of macrophages with pro-tumour functions.

The cleavage of matrix molecules is also essential to reveal binding sites previously masked from cell surface receptors. This is the case, for example, for MMP-2 which degrades collagen, unravelling integrin-binding sites that ultimately rescue melanoma cells from apoptosis.

Also, really interesting is the recent discovery that ECM fragments can modulate various functions of the innate immune cell, through the activation of TLRs[81]. For example, versican is able to activate TLR2 and TLR6 on TAMs and to stimulate the production of IL-6 and TNF, typical cytokines of cancer-related

inflammation. The matrix represents also a storage environment for growth factors (EGF, FGF, TGF-β family members, PDGF, VEGF) that are kept in an active form by binding ECM components. When the proteolytic activity increases, active growth factors are released, ultimately stimulating both tumour and stromal cells.

3. **Hypoxia and angiogenesis:** as previously described, hypoxic areas within a growing tumour, arise from an imbalance between oxygen supply vs oxygen consumption. This leads to a fall in the levels of pO_2 (partial pressure of oxygen) which in turn generates hypoxia, ultimately leading to the formation of new blood vessels, in a process called angiogenesis. These newborn vessels sustain the metabolic needs of tumour cells and together with the hypoxic environment, promote a malignant phenotype, typified by uncontrolled tumour growth and increased metastatic risk. Differently from normal cells, cancer cells do not adapt their metabolism to O_2 changes, rather favour glycolysis independently on the oxygen availability (the so-called 'Warburg effect'). This aerobic glycolysis is performed by the pyruvate kinase isoenzyme type M2 (M2-PK), the gene of which is HIF-1α dependent.

   The preferential location and site of accumulation of TAMs is represented by regions of the tumour poorly vascularized and hence poorly oxygenated. In these areas macrophages respond to the oxygen levels, via transcriptional upregulation of genes promoting mitosis, invasion, angiogenesis and metastasis. Obviously, the HIF-1α pathway plays a crucial role in the recruitment and activation of TAMs into solid cancers, as well as for the shaping of their pro-tumour functions.

4. **Immunosuppression:** TAMs release a wide array of factors (e.g. CSF-1, IL-4, IL-6, IL-10, PGE-2, TGF-β1,) that allow them to suppress the proliferation and cytotoxicity of NK- and T-cells. They also produce IL-10 and VEGF that prevent DCs maturation, impairing in this way efficient presentation of tumour antigens. Additionally, TAMs are a factory for the production of specific

chemokines like, CCL-2, CCL-22 and CCL-18 (**C**-**C** motif **L**igand-18). In particular, CCL-18 is an attractant for naïve T-cells, thereby promoting their accumulation in an environment dominated by M2-cells, which ultimately induces their anergy. A considerable contribution to immunosuppression is also provided by iron metabolism. In particular, high levels of intracellular heme in harness with induction of heme oxygenase, culminate in the production of CO (**C**arbon **M**onoxide), which has immunosuppressive activity.

1.10. Macrophages in melanoma

As for other cancers, also melanoma cells release molecules able to recruit macrophages to the tumour site. Additionally, malignant melanoma progression is accompanied by alterations in the population patterns of macrophages[82].

Several molecules are involved in macrophages-recruitment. Among them there are MCP-1 (**M**onocyte **C**hemoattractant **P**rotein-1) and VEGF-C. MCP-1, a potent macrophage-recruiting molecule, has been found expressed in human melanoma. It is also an enhancer of tumour angiogenesis and it accomplishes this function via the secretion of TNF-α, IL-1 and VEGF, through macrophage-recruitment, as well as by exerting autocrine/paracrine effects on melanoma cells. Nevertheless, MCP-1 can play a dual role, since its high level promotes tumour rejection, while low or intermediate levels, support tumour growth. On the other hand, VEGF-C also supports melanoma progression allowing interactions between tumour cells/lymphatic vessels, where it has been found overexpressed and also involved in macrophages-recruitment. As for MCP-1, also VEGF-C can have biphasic effects[82].

In melanoma, the M2-polarization status has been linked to the outcome of the patients. The macrophage M2-phenotype in melanoma has been showed to be induced by: (1) growth factors and additional molecules (i.e. ADM [**AD**reno**M**edullin] and CD73), (2) exosomes (both released by both melanoma cells and macrophages) and (3) T_{reg} cells.

(1) Among the growth factors, TGF-β1 and IL-10 play an important role. Notably, TGF-β1 can be also produced by M2-macrophages and has a crucial role in the polarization

of macrophages towards this phenotype[82,77]. Additionally, it has been reported that tumour cells producing high levels of TGF-β1, stimulate monocytes/macrophages ultimately supporting tumour growth and immune escape. Finally, TGF-β1 is also able to repress the release of nitric oxide from M1-macrophages, hence suppressing them. IL-10 can be also produced by melanoma cells (and not only by Th2 cells) and can modulate the immune responses, such as induction of M2-polarization[82]. In turn, M2-macrophages themselves, also release IL-10. In this way, they create a loop that, via this interleukin, allows the down-regulation of MHC-II-associated antigen presentation and expression of the co-stimulator molecule CD86 (also called B7-2) on macrophages which ultimately inhibits cytokine production by Th1 cells.

ADM instead is not a growth factor, but a multifunctional molecule playing a role in tumour angiogenesis in several cancers, including melanoma, where its levels and those of its receptor have been found increased (suggesting a role in melanomagenesis). Interestingly, in melanoma, TAMs have been identified as the major source of ADM. Via a paracrine mechanism - ADM induces phosphorylation of endothelial nitric oxide synthesis in endothelial cells - it promotes M2-polarization ultimately enhancing angiogenesis and hence melanoma growth[82].

The nucleotidase CD73, which is involved in the regulation of macrophage-infiltration, is upregulated in melanoma. CD73 also contributes to M2-polarization within the hypoxic regions of a tumour. In particular, reductions in the levels of CD73 have been observed in conjunction with downregulation of pro-M1-cytokines, a change that ultimately lead to a pro-neoplastic M2-phenotype and hence to tumour progression. Furthermore, the ecto-enzyme CD73 (together with CD36) can dephosphorylate extracellular ATP (**A**denosine 5′-**T**ri**P**hosphate) to adenosine, a potent anti-inflammatory and immunosuppressive molecule. ATP instead promotes pro-inflammatory effects on several immune cells. Hence the CD73/CD36 axis plays a key role in controlling immune modulation[83].

(2) In melanoma, a crucial role is also played by cancer cell- or stromal cell-derived exosomes (microvesicles with a diameter of 20-100 nm). Thanks to their size, exosomes are able not only to influence the TME locally, but also other distant cell types promoting a tissue environment favouring metastatic outgrowth. For example,

melanoma cell-derived exosomes affects the cytokine and chemokine profile of TAMs[84], while exosomes from macrophages can fuse with endothelial cells and deliver their cargo of both proteins and RNA to them, hence influencing the angiogenic response of the blood vessel[85]. (3) Both melanoma cells and macrophages can produce chemokines (e.g. macrophage-derived chemokines, MCP-1, …) contributing to T_{regs} migration. T_{reg} cells in turn, can promote the differentiation of monocyte into M2-macrophages. However, the T_{regs}/M2-macrophages interaction is mutually beneficial, since the phagocytes can directly induce the T_{reg} cells, which ultimately suppress tumour specific cytotoxic T-cell[82].

Other factors supporting melanoma growth and metastasis are angiotensin (which enhances the amplification of macrophages hence stimulating cancer-promoting immunity), IFN-γ, COX-2 (**C**yclo**OX**ygenase-2), a valid and reliable biomarker of melanoma progression) and IL-1β (the expression of which is associated with the degree of invasiveness and metastasis of melanoma)[82].

1.11. Macrophages and therapy

Specific therapies targeting macrophages are now emerging in the clinic. Several specific or indirect (i.e. not originally designed as macrophage-oriented) therapeutic approaches have been found to affect macrophage-activation and -polarization. An example is represented by inhibitors of the CSF-1 receptor (c-fms) kinase that have anti-angiogenic and anti-metastatic activity in models of cancer, such as acute myeloid leukaemia and melanoma models[86,87]. Another important point of macrophages-targeting therapy is to re-program their polarization status, since polarized phenotype have been observed to be reversible both *in vitro* and *in vivo*. However, achieving this reversal stably, while avoiding the side-effects of such reversal in long-term, has hindered this strategy's development[75].

As previously mentioned, cancer cells can also express high levels of CD47, which therefore represents a therapeutic target[88,89]. Indeed, antibody-mediated blockade of CD47 results in cancer cell elimination via two mechanisms: phagocytosis and

increased tumour antigen-specific T-cell activation, both of which are mediated by macrophages[90,91].

Overall, it can be concluded that it is crucial to understand the molecules and mechanisms involved in macrophages-plasticity and -polarization, in order to use them as therapeutic targets in cancer[75].

1.12. ICD (Immunogenic Cell Death)

As previously mentioned, in melanoma, BNIP3 expression levels correlates with those of CD47. However, CD47 a part from the several functions described above, also plays a relevant role in a particular form of cancer cell death with important consequences for the activation of anti-tumour immunity, called ICD.

[**The present paragraph has been adapted from the review article 'Immunogenic cell death', published in '*The International Journal of Developmental Biology*'**[92]].

Immunogenic cell death: the concept

Research published over the last decade has established that inciting a cancer cell death subroutine, associated with the activation of danger signalling pathways markedly increases the immunogenicity of the dying cells[93]. This cell death pathway has been termed ICD [93,94,95]. ICD has the ability to convert dying or dead cancer cells into a vaccine capable of inducing anti-cancer immunity in absence of any additional adjuvants[96]. A hallmark of ICD is the spatiotemporally defined emission of danger signals or DAMPs (**D**amage-**A**ssociated **M**olecular **P**attern**s**) as a part of an elaborate danger signalling module that helps in elevating the immunogenicity of dying cells.

ICD is highly stressor-dependent primarily because its execution requires simultaneous induction of ROS and ER stress[97]. The concomitant induction of oxidative stress and ER stress is crucial for elicitation of danger signalling pathways mediating the trafficking and emission of danger signals or DAMPs[98,99]. The first systematic screening for ICD inducers[100] recognised anthracyclines, such as mitoxantrone, as well as radiotherapy, as potent inducers of ICD in cancer cells. Since

this screening study, other novel and highly efficacious ICD inducers have been identified by targeted studies, which include various assorted chemotherapeutics, certain targeted therapeutics, various physical modalities, certain components of Chinese traditional medicine, certain oncolytic viruses and Hyp-PDT (**Hyp**ericin-based **P**hoto**D**ynamic **T**herapy)[101]. On the other hand, mitoxantrone is currently the most studied ICD inducer, in terms of both extensive *in vivo* immunological knowledge about its anti-cancer activity and its clinical immunostimulatory activity[93]. Mitoxantrone (or anthracyclines in general) has played an important role in characterizing the mechanics and kinetics of ICD[94,101].

Of note, the pioneering (as well as a large portion of the follow-up) research on ICD characterised it largely as an apoptotic yet immunogenic form of cancer cell demise[102,100]. However, it has now started to emerge that another programmed form of cell death i.e. programmed necrosis or necroptosis may also associate with ICD. It has been showed that NDV (**N**ewcastle **D**isease **V**irus) can induce ICD that is necroptotic in nature (since NDV-induced ICD is devoid of apoptotic features, yet is programmed, and can be inhibited by Necrostatin-1)[103]. However, the exact molecular pathways behind this NDV-induced necroptotic ICD still need further attention. Recently, using a genetic model to induce RIP3 (**R**eceptor-**I**nteracting **P**rotein kinase **3**)-mediated necroptosis in cancer cells it was showed that this form of regulated necrosis has the ability to elicit ICD in prophylactic vaccination settings. Necroptotic cancer cells were able to stimulate DC-mediated cross-priming of cytotoxic T cells and production of IFN-γ following tumour antigen stimulation[104]. In line with this, anthracyclines have been showed to trigger necroptosis in cancer cells expressing RIP3 and **MLKL** (**M**ixed **L**ineage **K**inase domain-**L**ike) (the key molecular effectors of necroptosis) featuring key hallmarks of ICD, in particular the release of ATP and HMGB1 (**H**igh **M**obility **G**roup **B**ox **1**), and eliciting T cell mediated anti-tumour responses *in vivo*[105].

Emission of danger signals: molecular determinants of immunogenic cell death

ICD has been found to associate with a spatiotemporally defined emission of three categories of danger signals or DAMPs, i.e. surface-exposed chaperones (exposed in the pre-apoptotic or pre-mortem stage), secreted or released nucleotides/nucleic acids (secreted/released in pre- or early-apoptotic/mortem stages) and release of endogenous TLR agonists (released in mid- or post-apoptotic/mortem stages[94,76,87]).

- ***Surface exposure of ecto-CALR***

Surface exposure (ecto-) of CALR has been found to be crucial for the immunogenicity of dying or dead cancer cells. Ecto-CALR binds to CD91 receptor on immune cells thereby exerting its function as an 'eat me' signal[55,98,100,88]. Moreover, ecto-CALR is capable of inciting the production of both IL-6 and TNF from DCs thereby facilitating Th17 polarization[106,107]. Indeed, exogenously injected CALR has been observed to promote tumour lymphocyte infiltration and increase responsiveness to cancer immunotherapy[108]. Interestingly, tumoural $CALR^{high}$-phenotype has been observed to be predictive of positive clinical responses to therapy with only ICD inducers like radiotherapy or paclitaxel in non-small cell lung or ovarian cancer patients, respectively (but not non-ICD inducer like topotecan in ovarian cancer or untreated non-small cell lung cancer patients)[109]. Elevated levels of phosphorylated eIF2α (**e**ukaryotic **I**nitiation **F**actor **2** **α**) have been showed to correlate with higher ecto-CALR on malignant myeloblasts from patients with acute myeloid leukaemia regardless of chemotherapy; and higher ecto-CALR correlated with ability of autologous T cells to secrete IFN-γ on stimulation with blast-derived dendritic cell and better overall survival of the patients[110]. This observation suggests that ER stress-regulated ecto-CALR is associated with the stimulation of anti-cancer immunity and may harbour a prognostic role in cancer patients.

- ***Secretion or release of ATP***

Active secretion of the nucleotide, adenosine triphosphate (ATP)[111] from dying cells is essential for ICD[112,113] and anti-tumour immunity[114].

Secreted ATP, is a potent short-range 'find me' signal[111], that can bind ionotropic (P2X) as well as metabotropic (P2Y) purinergic receptors[111,112]. Secreted ATP has also been observed to help in DC activation[112]. During ICD, secreted ATP has been reported to bind to the P2X7 receptors, causing activation of the NLRP3 (**N**acht, **LR**r and **P**yd domains-containing protein **3**) inflammasome[112], which in turn mediates caspase-1-mediated processing and secretion of IL-1β, a crucial cytokine in the stimulation of anti-tumour immunity[112]. ICD-based secreted ATP has also been reported to mediate intra-tumoural recruitment and differentiation of antigen presenting $CD11c^{+}CD11b^{+}Ly6c^{hi}$ cells[115].

- ***Secretion or release of HMGB1***

Release of HMGB1, which binds to TLR4 or TLR2 on immune cells, has been found to be critical for ICD[116]. During ICD, released HMGB1 binds TLR4 on DCs and, on one hand, activates production of pro-inflammatory cytokines yet on the other hand assists in proper antigen-presentation[75,117]. Interestingly, extracellular HMGB1 has also been found to suppress the activity of the immunosuppressive T_{reg} cells[118].

However, there are several contradictory observations regarding the role of HMGB1 in tumourigenesis, because HMGB1 can exhibit a redox-dependent switch in functionality or exert effects on other stromal cells (e.g. endothelial cells) rather than specific effects on immune cells, thereby making it hard to draw steadfast conclusions[119,120].

Danger signalling pathways: mechanistic determinants of immunogenic cell death

The ability of ICD-inducing therapies to cause emission of DAMPs associates with their capability of activating danger signalling in the stressed or dying cancer cells. During ICD, the emission of DAMPs by the stressed or dying cells occurs in spatiotemporally-defined fashion, largely governed by the activation of danger signalling pathways, during which stressed/dying cells surface expose CALR in the pre-apoptotic phase (e.g. before phosphatidylserine externalization), actively secrete

ATP (either during pre-apoptotic stage or during the blebbing phase) and ultimately release HMGB1, post-apoptotically, e.g. during secondary necrosis. Here, secondary necrosis is an autolytic process of cellular disintegration following apoptosis entailing release of cellular components. It occurs when the apoptotic program has reached its terminal stage but the apoptotic cell hasn't been cleared by a phagocyte (a largely *in vitro* phenomena that can also happen *in vivo* in exceptional circumstances when the phagocytic capacity is exceeded due to major trauma or damage).

The requirement for the co-existence of threshold levels of ROS and ER stress for the activation of the danger signalling machinery eliciting ICD, has been established in different studies[78,112], which demonstrated that the immunogenicity of the dying cells is severely compromised in the presence of anti-oxidants or in the absence of a robust ER stress response.

It should be mentioned that although ecto-CALR may also occur in apoptotically dying cells as a result of general exposure of ER chaperones and ER and Golgi membranes on the cell surface[101], during ICD, surface exposure of CALR is an active process that precedes the morphological signs of apoptosis. Elegant studies have showed that CALR exposure in response to anthracyclines, like doxorubicin and mitoxantrone, requires cotranslocation of ERp57 and relies on a complex pathway consisting of the sequential activation of three signalling modules: (a) an ER stress-ROS module consisting of the activation of the PERK (protein kinase R (**P**KR)-like **E**ndoplasmic **R**eticulum **K**inase)-eIF2α axis; (b) an apoptotic module, reliant on the pre-apoptotic caspase-8-mediated cleavage of BAP31 (**B**-cell receptor-**A**ssociated **P**rotein **31**) and regulated by Bax/Bak and Ca^{2+} signals; and (c) a SNARE (**S**oluble **N**SF **A**ttachment **RE**ceptor)-dependent, ER-to-Golgi anterograde transport[97]. Furthermore, a systematic comparison of the molecular effectors of the DAMP trafficking mechanisms in dying cancer cells in response to anthracyclines or Hyp-PDT, revealed that ecto-CALR following Hyp-PDT occurs independently of ERp57 co-translocation and relies distinctively on PERK-regulated secretory pathway, Bax/Bak and an intact ER-to-Golgi anterograde transport, whereas, in contrast to anthracyclines, is independent of eIF2α phosphorylation, Ca^{2+} and caspase-8, or caspase signalling in general[79,121]. This study revealed also that both ICD inducers require a PI3K

(**P**hospho**I**nositide **3**-**K**inase)-mediated distal secretory pathway to emit ecto-CALR[98]. Importantly, in the case of Hyp-PDT, active ATP secretion occurred concomitantly to ecto-CALR and was governed by similar PERK-regulated proximal secretory pathway and PI3K-dependent exocytosis mechanisms[98].

This finding is also interesting considering that the active release of ATP from dying cells in response to UV, anti-CD95/FAS (**F**irst **A**poptosis **S**ignal**)** therapy[111] and chemotherapy[122] usually occurs during the early-intermediate phases of apoptosis (e.g. blebbing phase) and involves pannexin-1-dependent[111] or autophagy-dependent ATP secretion pathways[122], which are both reliant on caspase activity. In the case of mitoxantrone and oxaliplatin, ATP release from dying cancer cells was showed to be inhibited by the knockdown of essential autophagy-related genes (*ATG5*, *ATG7* and *BECN1* [**BEC**li**N1**]), such that reducing extracellular ATP levels during ICD compromised tumour-specific immune response *in vivo*[113]. However, the role of autophagy in the modulation of danger signalling during ICD appears to be also largely context-dependent. While chemotherapy-induced ICD exploits the trafficking properties of the autophagy machinery to mobilize ATP, but does not affect ecto-CALR[113]; autophagy during Hyp-PDT acts apically as a cytoprotective degradation mechanism that assists in the removal of the excessive amount of oxidized proteins (including ER chaperones such as CALR) generated by Hyp-PDT, thereby reducing ecto-CALR, without affecting the trafficking and release of ATP[90,123,124]. These observations reveal the overall complexity of ATP secretion pathways.

Hence, all together these studies indicate that the danger signalling underlying the mobilization of crucial DAMPs, like ecto-CALR and secreted ATP, in response to anti-cancer therapy is subjected, at least in part, to the type of the cell death stimulus and the molecular nature and mediators of the danger signalling it instigates. This notion needs to be considered in future clinical studies when anti-cancer agents may be combined with modulators of cell stress pathways, such as autophagy, which are clinically available.

Immunological and operational determinants of immunogenic cell death

- ***Tumour-rejecting immunity in a vaccination context***

Almost a decade-long list of published research has characterised various molecular, mechanistic and immunological determinants of ICD; however, the gold-standard approach to evaluate the ability of a specific agent or modality to elicit *bona fide* ICD still relies on vaccination assays showing tumour-rejecting capabilities of the immunized host[75,102]. In this setting, the cancer cells are treated *in vitro* with the anti-cancer therapy under consideration and then injected either intraperitoneally or subcutaneously/intradermally into the flank of immunocompetent syngeneic rodents, followed by a challenge with living cells of the same cancer type into the opposite flank or another site-of-choice for the tumour. The proportion of rodents that resist such subcutaneous tumours reveals the degree of immunogenicity of cell death for the therapy under consideration (in case of prototypical ICD inducers like anthracyclines or Hyp-PDT, this happens for 70-90% of mice, although strain-to-strain, cancer type-to-cancer type and setup-to-setup differences can exist)[98,81,125].

- ***Innate and adaptive immune system stimulation***

DAMPs associated with ICD exert strong immunostimulatory effects upon binding to their respective receptors on immune cells, like PRR (**P**attern **R**ecognition **R**eceptors), scavenging receptors or purinergic receptors expressed by immune cells[126]. DAMPs and other immunomodulatory signals brought about by the ICD inducers helps them in establishing a productive interface between dying/dead cancer cells and the immune system.

The interface between cancer cells undergoing ICD and the immune system has been analysed in details, for both mice as well as human settings, for only a hand full of ICD inducers which include mainly anthracyclines or oxaliplatin, radiotherapy and Hyp-PDT. These studies have showed that, cancer cells undergoing ICD are capable of attracting DCs and certain specific DC subsets mainly *via* secreted ATP (but possible also *via* HMGB1). Once attracted, DCs interacting with cancer cells experiencing ICD undergo efficient stimulation, differentiating into fully mature immunogenic DCs

overexpressing various surface maturation markers like CD80, CD83, CD86 and MHC-II accompanied by production of immunostimulatory cytokines[79,81,102,107,109,110]. This APC/DC-level activations typically culminates into the recruitment of T cells followed by their activation[90,127,128,129,130]. Of note, most of the above results are derived either from *ex vivo* immune cell co-culture experiments or analysis of heterotropic subcutaneous tumours. In future there is an urgent need to extend these studies to either spontaneous tumour models or orthotopic tumour models.

2. Aims and Objectives

As previously discussed, BNIP3 has been found to support crucial pro-melanoma functions, ranging from clonogenic growth to mitochondrial viability. Despite the discovery of its intrinsic properties, there is still lack of knowledge about the impact of BNIP3 on immunological processes in this cancer type. Considering also that melanoma-associated BNIP3 affects the expression levels of the 'don't eat me' signal CD47, the aim of the present work was to study the most foundational immunological processes, namely phagocytosis and chemotactic recruitment, both *in vitro* and *in vivo*. Moreover, it was also assessed whether melanoma-associated BNIP3 could have had any effect on key determinants of ICD (induced by the chemotherapeutic, MTX [**M**i**T**o**X**antrone]), particularly danger signals, as well as *in vivo* anti-cancer vaccination.

Therefore, the main aims of the present work were the following:

1. *In vitro* analysis of the role of melanoma-associated BNIP3 in regulating phagocytosis of melanoma cells by macrophages;
2. *In vivo* investigation of the ability of melanoma cells (from the vantage point of BNIP3 levels) to affect recruitment of, polarization of and phagocytosis by macrophages;
3. *In vitro* study of the effect of melanoma-associated BNIP3 on ICD-derived danger signals;
4. *In vivo* examination of the role of melanoma-associated BNIP3 in ICD-mediated anti-cancer vaccination.

The above mentioned milestones were addressed by means of various multidisciplinary and complementary analyses, ranging from flow cytometry-based analysis of phagocytosis (on cell lines and from the peritoneum of mice) to immunophenotyping of macrophages (from mice peritoneum and subcutaneous tumours). Also *in vitro* assays were performed to test the effects of BNIP3 on the essential danger signals emitted by chemotherapy-induced ICD, together with *in vivo*

anti-cancer vaccination experiments, using prophylactic vaccination strategy involving MTX-induced dead/dying melanoma cells.

These aims are very crucial, in order to understand the cancer cell non-autonomous role of BNIP3 in modulating the immunological interface. Being a Bcl-2 family member and a BH3-only protein, BNIP3 could be a potential drug target in the near future. By addressing the above aims, it would be possible to understand the positive or negative consequences of BNIP3-targeting for anti-melanoma immunity. This information can be eventually utilized to design proper combinatorial regimens against melanoma.

3. Results

[The present section has been adapted from the accepted research paper 'BNIP3 modulates the interface between B16-F10 melanoma cells and immune cells' published in the multi-disciplinary journal, '*Oncotarget*'[131]]

BNIP3 modulates the interface between B16-F10 melanoma cells and immune cells

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The hypoxia responsive protein BNIP3, plays an important role in promoting cell death and/or autophagy, ultimately resulting in a cancer type-dependent, tumour-enhancer or tumour-suppressor activity. We previously reported that in melanoma cells, BNIP3 regulates cellular morphology, mitochondrial clearance, cellular viability and maintains protein expression of CD47, a pro-cancerous, immunosuppressive 'don't eat me' signal. Surface-exposed CD47 is often up-regulated by cancer cells to avoid clearance by phagocytes and to suppress immunogenic cell death (ICD) elicited by anticancer therapies. However, whether melanoma-associated BNIP3 modulates CD47-associated immunological effects or ICD has not been explored properly. To this end, we evaluated the impact of the genetic ablation of BNIP3 (i.e. $BNIP3^{KD}$) in melanoma cells, on macrophage-based phagocytosis, polarization and chemotaxis. Additionally, we tested its effects on crucial determinants of chemotherapy-induced ICD (i.e. danger signals), as well as *in vivo* anticancer vaccination effect. Interestingly, loss of BNIP3 reduced the expression of CD47 both in normoxic and hypoxic conditions while macrophage phagocytosis and chemotaxis were accentuated only when $BNIP3^{KD}$ melanoma cells were exposed to hypoxia. Moreover, when exposed to the ICD inducer mitoxantrone, the loss of melanoma cell-associated BNIP3 did not alter apoptosis induction, but significantly prevented ATP secretion and reduced phagocytic clearance of dying cells. In line with this, prophylactic vaccination experiments showed that the loss of BNIP3 tends to increase the intrinsic resistance of B16-F10 melanoma cells to ICD-associated anticancer vaccination effect *in vivo*. Thus, normoxic vs. hypoxic and live vs. dying cell contexts influence the ultimate immunomodulatory roles of melanoma cell-associated BNIP3.

Oncotarget. 2018; 9:17631-17644. doi.org/10.18632/oncotarget.24815

3.1. BNIP3 and hypoxia regulate the phagocytosis of B16-F10 melanoma cells by J774 macrophages

In order to systematically decipher the role of cancer cell-associated BNIP3 in regulating the melanoma-immune cell interface, we knocked-down the overall expression of BNIP3 via the shRNA methodology ($BNIP3^{KD}$), in the well-established murine B16-F10 melanoma cells (***Figure 6A***). Additionally, to account for the prominent status of BNIP3 as a hypoxia-inducible molecule[132], we compared the effects of normoxia (20% O_2) with hypoxia (1.5% O_2) on the respective B16-F10 cells. Notably, the knock-down of BNIP3 was prominent under both normoxic and hypoxic conditions, which, as expected, elevated the protein levels of BNIP3 only in the B16-F10 cells expressing control shRNA (i.e. $BNIP3^{WT}$; ***Figure 6A***). Interestingly, $BNIP3^{WT}$ B16-F10 cells exposed to hypoxia, significantly (*p = 0.0411) up-regulated the surface levels of CD47 (i.e. ecto-CD47) as compared to the ones exposed to normoxia (***Figure 6B***). The latter observation aligns with the published literature demonstrating the immunosuppressive effects of hypoxia[133]. Notably, $BNIP3^{KD}$ B16-F10 cells not only exhibited a significant (**p = 0.0043) reduction in ecto-CD47 in normoxic conditions, as compared to $BNIP3^{WT}$ cells (in keeping with our published report[29]), but also displayed a tendency to dampen ecto-CD47 levels under hypoxia, albeit non-significantly (***Figure 6B***; p = 0.3052).

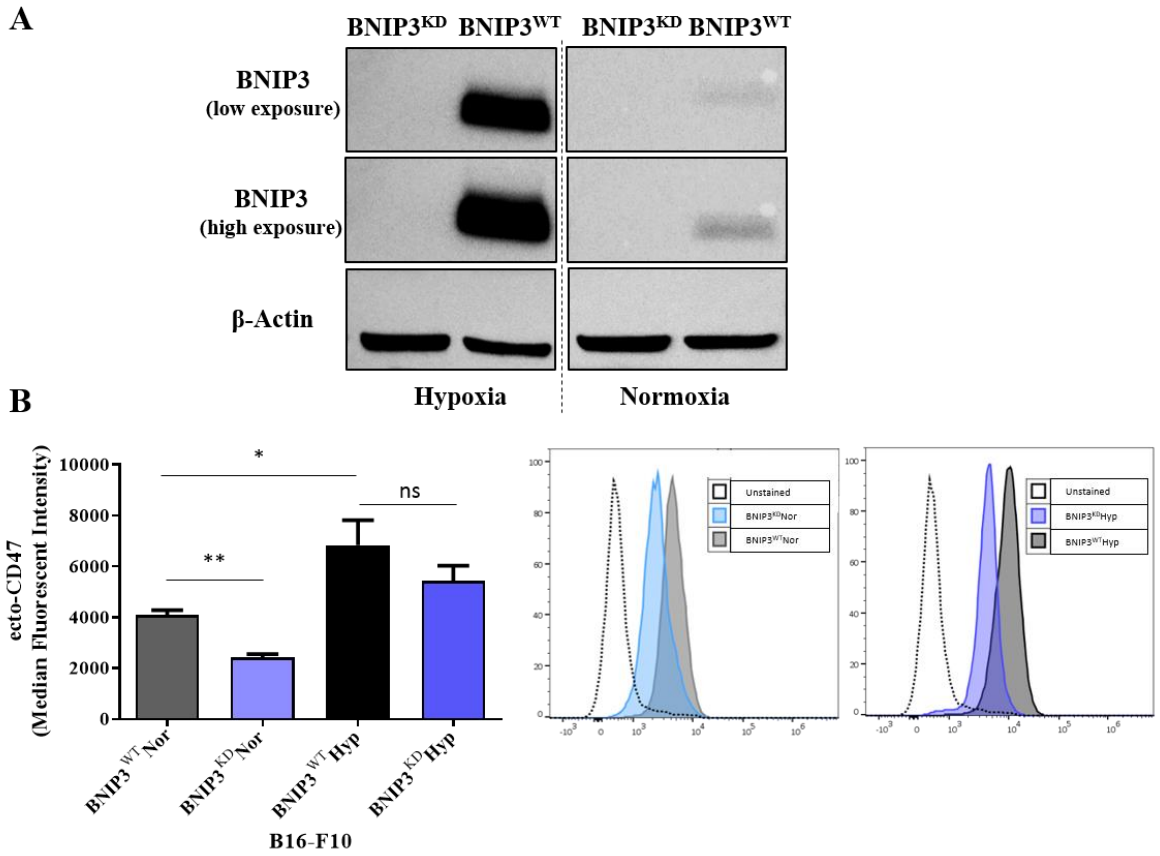


Figure 6 (A-B): BNIP3 and hypoxia modulate CD47 expression in B16-F10 melanoma cells. (**A**) Representative western blot for the efficiency of BNIP3 knock-down in B16-F10 cells after 24 h treatment under normoxic and hypoxic conditions (both low and high exposure are reported). Actin was used as loading control. (**B**) Flow Cytometry-based quantification (left panel) and representative histograms (right panel, fluorescence intensity) of the level of surface CD47 (ecto-CD47) in B16-F10 cells ($BNIP3^{WT}$ and $BNIP3^{KD}$) after 24 h of culture in normoxia (Nor) or hypoxia (Hyp). Data expressed as mean ± SEM and analysed with Mann-Whitney's *t*-test (***p* = 0.0043, **p* = 0.0411, ns = not significant [*p* = 0.3052]) as indicated by the bar, *n* = 3 independent experiments).

To understand the impact of this interplay between BNIP3-hypoxia-CD47 on the phagocytic clearance of melanoma cells by macrophages, we performed *in vitro* phagocytosis experiments. These involved co-incubation of B16-F10 cell lines with the macrophage-like J774 cells followed by flow cytometric scoring of phagocytosis[134,135]. Notably, $BNIP3^{KD}$ B16-F10 cells, under normoxic settings, did not undergo efficient phagocytic clearance by the J774 cells (***Figure 6C***). This was surprising, considering the significantly reduced ecto-CD47 levels exhibited by these cells (***Figure 6B***). Alternatively, when the cells were exposed to hypoxia, the phagocytic clearance of

BNIP3^{WT} B16-F10 cells by J774 cells was increased significantly (***p = 0.0007, as compared to normoxic-BNIP3^{WT} cells; ***Figure 6C***), thereby indicating the presence of phagocytic determinants other than CD47 in this setting. Moreover, this hypoxia-stimulated phagocytosis of living melanoma cells was further potentiated by BNIP3^{KD} in a statistically significant manner (***p = 0.0005, as compared to normoxic-BNIP3^{WT} cells; ***Figure 6C***). In fact, the phagocytosis of BNIP3^{KD} cells under hypoxia was also significantly higher than BNIP3^{WT} cells under hypoxia (*p = 0.0172, ***Figure 6C***).

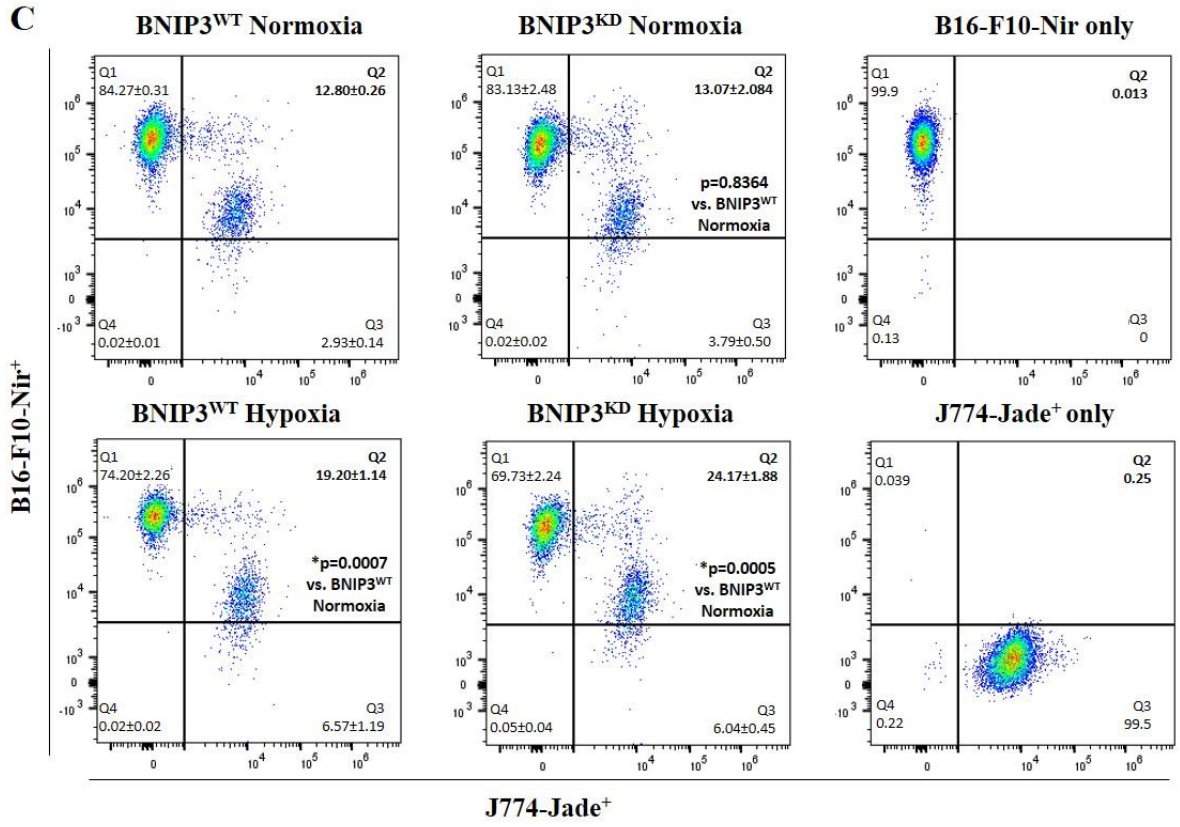


Figure 6C: BNIP3 and hypoxia modulate the phagocytosis of B16-F10 melanoma cells by macrophages.
(C) J774-mediated phagocytosis of B16-F10 after 24 h co-incubation, based on flow cytometry data with relative gating strategy. The engulfing ability is reported in Q2 as percentage of B16-F10-Nir^{+} / J774-Jade^{+} cells. Data expressed as mean ± SD analysed with Student's t-test and p-value, with respect to BNIP3 Nor, is reported in Q2 quadrant (These dot plots are representative of independent experiments; n = 3).

Note that the viability of B16-F10 cells was assessed *a priori* through a cell death assay, based on exclusion dye staining via propidium iodide (PI). Results are shown in the addendum (***page 102***).

Next, we analysed the ecto-CALR levels in BNIP3^{WT} or BNIP3^{KD} melanoma cells (relative to normoxia or hypoxia). FACS analysis using an anti-CALR antibody did not reveal any significant exposure of this molecule in the above conditions (***Figure 7***). This ruled out the possibility that differences in ecto-CALR might be counter-balancing the susceptibility of melanoma cells to undergo phagocytosis in the above settings. Thus, B16-F10 melanoma-specific BNIP3 ablation in combination with hypoxia, stimulates phagocytic clearance of melanoma cells by macrophages, although this does not inversely correlate with the ecto-CD47 levels.

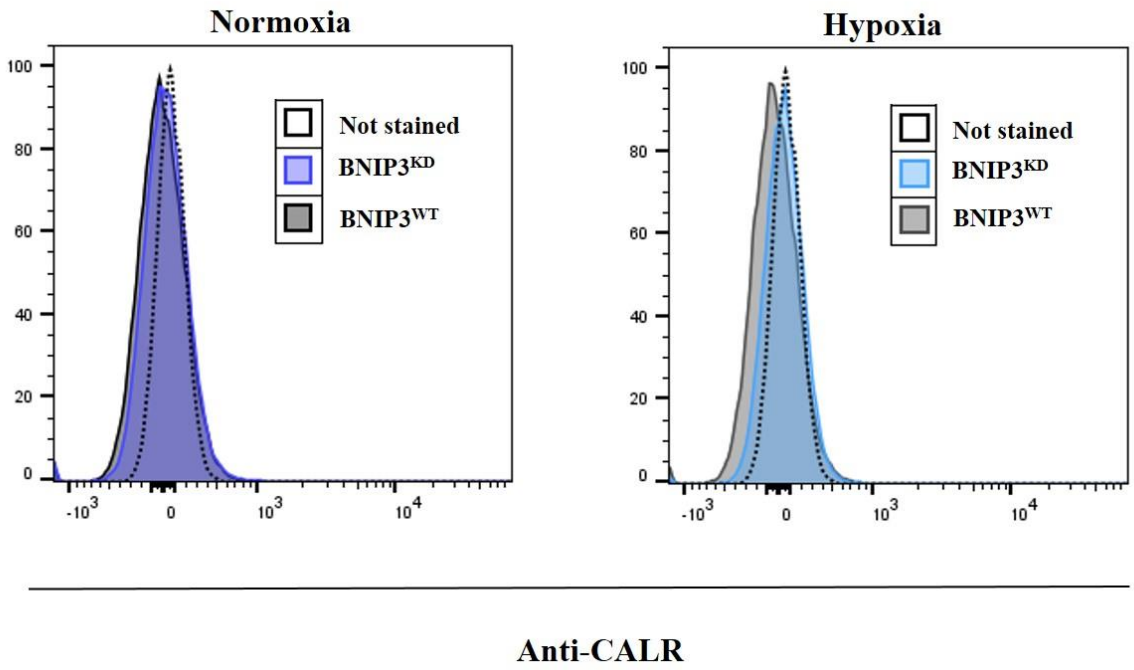


Figure 7: Levels of ecto-CALR on BNIP3^{WT} and BNIP3^{KD} B16-F10 cells kept 24 h under normoxia or hypoxia (representative histograms).

3.2. BNIP3 does not alter the ability of melanoma cells to recruit, polarize or be phagocytosed by macrophages *in vivo*

In a tissue context, efficient phagocytic clearance of damaged cells at a 'wounding site' is carried out by local as well as newly recruited macrophages[136]. In the latter case, a 'wounding site' is typically flagged by various chemotactic factors, which can be sensed by macrophages for directional chemotaxis[119,137]. Of note, B16-F10 cells have been widely reported to modulate the chemotactic recruitment of different immune cells, including macrophages[137,138]. To this end we wondered whether BNIP3, also in light of its dominant functions in cytoskeleton regulation[29], may affect the secretion of pro- or anti-macrophage chemokines. To address this, we initially examined the release of major chemokines by BNIP3^{WT} and BNIP3^{KD} cells, relative to normoxic or hypoxic conditions, by using an antibody array followed by a volcano plot-based representation. Although to an insignificant level, BNIP3^{KD} in B16-F10 cells (either alone or in combination with hypoxia), exerted diverse (and sometimes even contradictory) effects on the secretion of various chemokines and cytokines, with disparate functionalities (***Figure 8***). More specifically under normoxic conditions (***Figure 8B***), BNIP3^{KD} B16-F10 cells secreted increased amounts of the pro-myeloid cell chemokines i.e. CCL-2 (a potent macrophage-recruiting chemokine) and CXCL-1 (chemokine [**C**-**X**-**C** motif] **L**igand-**1**], a potent neutrophil recruiting chemokine) (***Figure 8B***). Yet in the same setting, some chemokines with the ability to attract myeloid cells (CX3CL-1 [chemokine (**C**-**X3**-**C** motif) **L**igand-**1**], CXCL-10), macrophages (CCL-3, [**C**-**C** motif **L**igand-3]) or lymphocytes (CX3CL-1, CXCL-16 - chemokine [**C**-**X**-**C** motif] **L**igand-16-, CXCL-10) exhibited strong reduction in secretion (***Figure 8B***). Normoxic BNIP3^{KD} B16-F10 cells also exhibited reduction in secretion of certain pro-inflammatory cytokines like GM-CSF and IL-1α (***Figure 8B***). Curiously, hypoxic treatment of BNIP3^{KD} B16-F10 cells largely diminished the above patterns observed with normoxic BNIP3^{KD} B16-F10 cells (except for CXCL-10) (***Figure 8C***). However, hypoxic BNIP3^{KD} B16-F10 cells did secrete increased amounts of the pro-macrophage chemokine, CCL-3, and the pro-inflammatory cytokine, IL-1β (***Figure 8C***)[139].

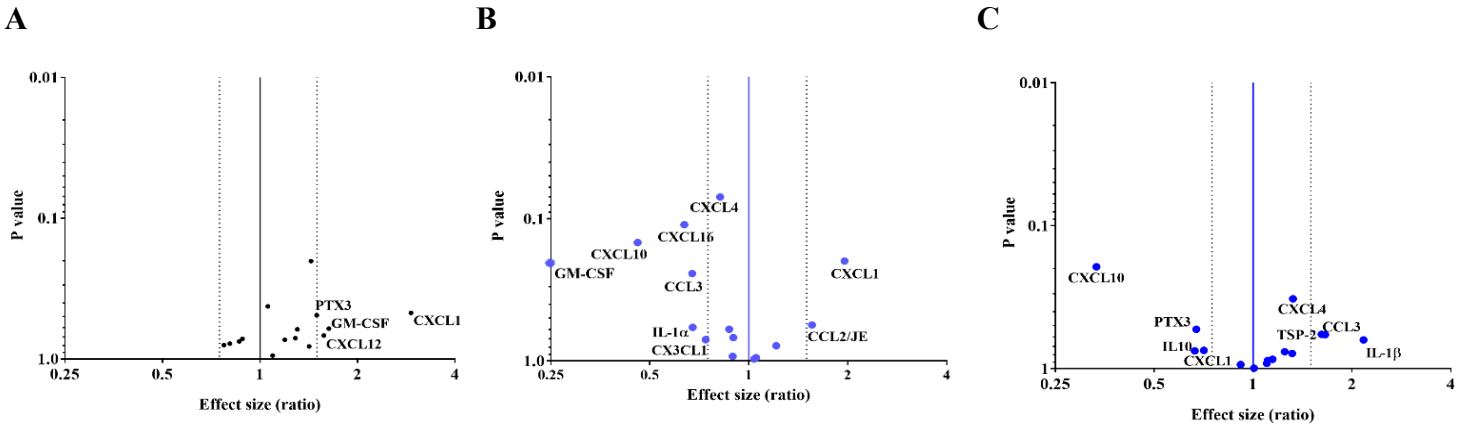


Figure 8: BNIP3 and hypoxia differentially regulate secretion of pro-macrophage chemokines and pro-inflammatory cytokines by B16-F10 melanoma cells.
Volcano plots depicting the changes (indicated by the increased or decreased effect size [ratio]) in the major murine chemokines detected in the secretome of BNIP3^{WT} and BNIP3^{KD} B16-F10 cells measured with an antibody array. The conditions compared are: (A) BNIP3^{WT} under hypoxia versus normoxia; (B) BNIP3^{KD} versus BNIP3^{WT} all under normoxia; (C) BNIP3^{KD} versus BNIP3^{WT} all under hypoxia. The 1.5 effect size (ratio) increase or 0.75 effect size (ratio) decrease in secretion/release is indicated by the dotted lines (n = 3, Student's t-test).

Next, to understand whether these results might translate into differential recruitment (and polarization) of macrophages *in vivo*, we utilized the murine peritoneal injection model, a well-established model to study myeloid chemotactic recruitment towards dying cells[140]. Herein, we injected the BNIP3^{WT} or BNIP3^{KD} B16-F10 cells, pre-treated or not with hypoxia, in the mice peritoneum. This was followed by flow cytometry-based analyses of peritoneal lavage-derived CD11b^{+}F4/80^{+} macrophages (***Figure 9A***; ***see page 71***). Here, only hypoxic BNIP3^{WT} B16-F10 cells caused some, albeit insignificant (***Figure 9B***; p = 0.50; ***see page 71***), increase in macrophage-recruitment. However, this increase was abolished in mice injected with hypoxic melanoma cells harbouring BNIP3^{KD} (***Figure 9B***; p = 0.62 vs. hypoxic BNIP3^{WT} cells; ***see page 71***).

We also analysed the phenotypic polarization status of peritoneal macrophages possibly interacting with the injected melanoma cells, owing to some interesting patterns observed above with pro-inflammatory cytokines. Macrophages tend to exist in various states of polarization depending on the context. However there are three major polarization statuses i.e. M0 (naive, unpolarised macrophages; marked by

$CD86^{low}MHC\text{-}II^{low}CD206^{low}CD11b^{+}F4/80^{+}$), M1 (pro-inflammatory macrophages; marked by $CD86^{high}MHC\text{-}II^{high}CD11b^{+}F4/80^{+}$) and M2 (anti-inflammatory macrophages; marked by $CD206^{high}CD11b^{+}F4/80^{+}$) (***Figure 9A***; ***see page 71***)[141]. In our settings, injection of different melanoma cell lines did not cause any statistically significant increase in the M1-polarization markers on the peritoneal lavage-derived macrophages (***Figure 9C***; ***see page 71***). Of note, we did observe a trend towards decrease in M1-macrophage-polarization amongst mice injected with hypoxia pre-conditioned B16-F10 cells (irrespective of BNIP3 status), yet these trends were not statistically significant. On the level of M2-polarization markers however, the combination of $BNIP3^{KD}$ in B16-F10 cells and hypoxic pre-conditioning, did potentiate (to a small, albeit insignificant extent; $p = 0.40$) the emergence of M2-macrophages (***Figure 9D***; ***see page 71***). However, these macrophage-recruitment and -polarization trends were not accompanied by any palpable increase in phagocytosis of (normoxic or hypoxic pre-conditioned) $BNIP3^{KD}$ B16-F10 cells by the peritoneum-resident macrophages (***Figure 10***; ***see page 71***).

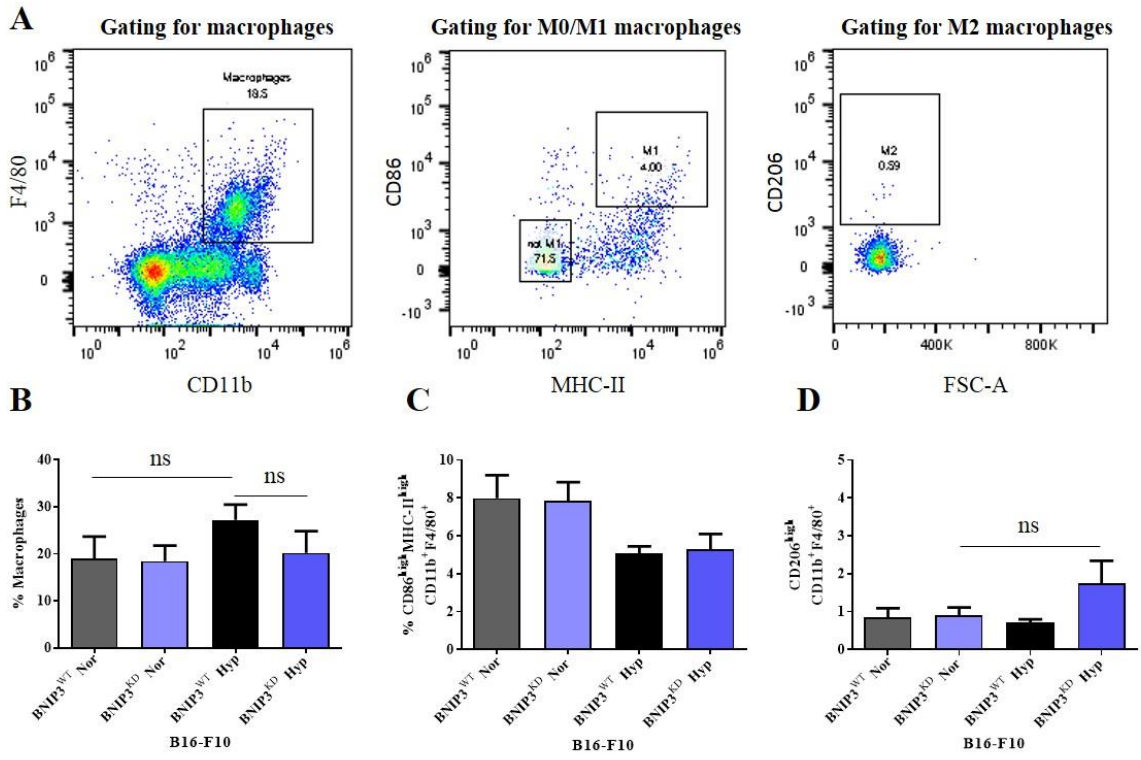


Figure 9: Ablation of BNIP3 and/or hypoxic pre-conditioning in melanoma cells has trivial consequences for the recruitment and polarization of macrophages in vivo.
(**A**) Gating strategy for the flow cytometry-based identification of the macrophage population and its polarization statuses. Quantification of (**B**) the recruitment of macrophages ($F4/80^{+}CD11^{+}$) in the peritoneum of mice, following injection of $BNIP3^{WT}$ and $BNIP3^{KD}$ B16-F10 cells kept under normoxia or hypoxia conditions, and their polarization towards (**C**) M1 ($CD86^{high}MHC\text{-}II^{high}$) or (**D**) M2 ($CD206^{high}$) phenotype expressed as mean ± SEM (n = 6 mice/condition), all analysed with one-way ANOVA (ns = not significant; p-values from left to right: $p = 0.50$, $p = 0.62$ and $p = 0.40$).

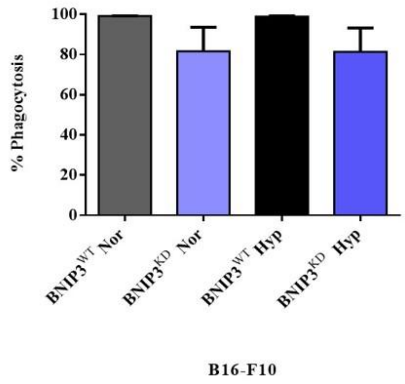


Figure 10: Phagocytosis of $BNIP3^{KD}$ B16-F10 cells is not increased in vivo.
Quantification via flow cytometry of $BNIP3^{KD}$ or $BNIP3^{WT}$ B16-F10 cells phagocytosed by intraperitoneal macrophages in C57BL/6 mice after 24 h pre-conditioning in normoxia or hypoxia. The engulfing ability was expressed as percentage of $F4\text{-}80^{+}/CD11b^{+}/pHRodo^{+}$ cells (mean ± SEM, mice = 6/group, Student's t-test).

3.3. Melanoma-associated BNIP3 affects tumour growth in an immune-competent *in vivo* context

Although the murine peritoneal injection model is a well-established model to study chemotaxis, it is not suitable to investigate the effects of BNIP3 within a tumoural context, because it does not take into account the signals generated within a TME, which can be highly influential in dictating anti-melanoma immune responses.

To address this question, we utilised a syngeneic mouse model where B16-F10 melanoma cells, either control or $BNIP3^{KD}$, were subcutaneously injected in C57Bl/6J mice and tumour growth was monitored over time. The growth of $BNIP3^{KD}$ B16-tumours was significantly delayed compared to that of $BNIP3^{WT}$ tumours (***Figure 11***). This result suggested a significant role for melanoma-associated BNIP3 in regulating *in vivo* tumour progression and created a potential scenario for *in vivo* contribution of immune cells in influencing tumour growth.

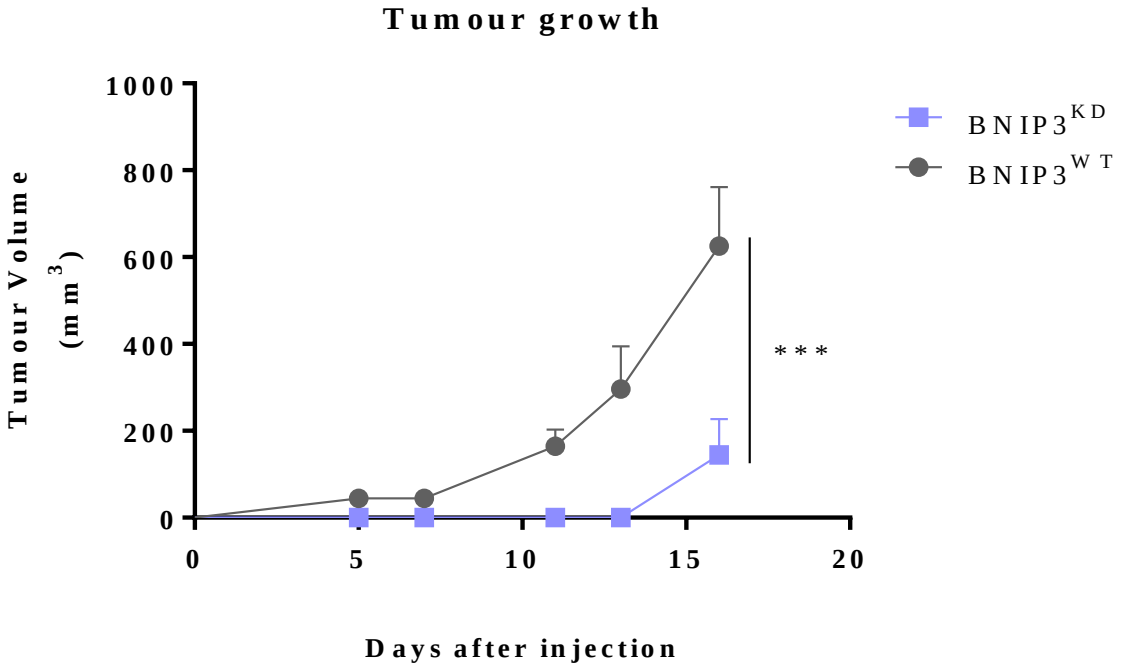


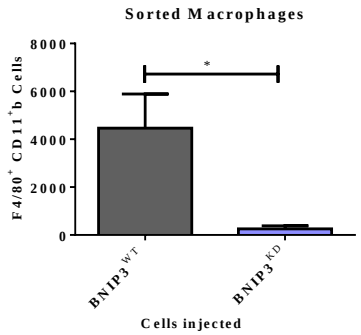
Figure 11: Tumour growth in the in-vivo immune-competent, syngeneic, murine system.

B16-F10 mouse melanoma cells bearing non-targeting shRNA ($BNIP3^{WT}$) or shRNA directed BNIP3 ($BNIP3^{KD}$) were subcutaneously transplanted into immune-competent syngeneic C57BL/6 mice. Primary tumour growth of BNIP3 deficient or proficient B16-F10 tumours ($BNIP3^{WT}$: n = 8; $BNIP3^{KD}$: n = 8), as assessed by calliper measurements of tumour volume. Data expressed as mean ± SEM) and analysed with Student's *t*-test (***$p < 0.05$).

3.4. Melanoma cells lacking BNIP3 exhibit defects in the recruitment of tumour associated macrophages (TAMs)

The above defects in tumour growth exhibited by BNIP3-ablated melanoma cells were intriguing. In light of the differences in phagocytosis of BNIP3^{KD} cells observed under hypoxia and the tumour growth outcomes, we wondered whether TAMs might be playing some role in influencing the growth of BNIP3-ablated melanoma cells *in vivo*. The immunoprofile of melanoma has been extensively characterised on the level of T cells and DCs[142]. However, the role of TAMs in melanoma remains controversial since on one hand TAMs can fuel melanoma growth, yet on the other, they have the ability to eradicate the neoplastic cells. The final outcome is dictated by the type of molecules involved in the interactions between macrophages and melanoma cells[142]. Interestingly, we found that BNIP3^{KD}-tumours displayed a reduced amount of CD11b^{+}/F4-80^{+} macrophages (***Figure 12A***). Considering the impressive reduction in the number of recruited TAMs observed for BNIP3^{KD}-tumours we moved towards the analysis of the M1/M2-polarization status of this population. The analysis of M1/M2-markers at both surface (for phenotypic markers like CD86, MHC-II and CD206) and mRNA level (for immunomodulatory cytokines like CXCL-10, IL-12α, IFN-β [**I**nter**F**ero**N**-**β**], IL-10 as well as for IDO-1 [**I**ndoleamine 2,3-**D**i**O**xigenase-**1**], Nos2 [**N**itric **o**xide **s**ynthase **2**] and Arg1) did not provide evidence for a defined M1 or M2-polarization status of TAMs, but rather disclosed the presence of a M0 population i.e. largely unpolarised/immature TAMs in the tumours (***Figure 12B, C***). This global cell sorting analysis, suggests that melanoma associated-BNIP3 affects predominantly the recruitment and/or the survival of TAMs within the growing tumours rather than their phenotypic polarization. Furthermore, the reduced TAMs accumulation observed in BNIP3^{KD}-tumours compared to control melanomas, may also be caused by changes in the TME impeding the infiltration and/or the survival of TAMs into the tumour.

A



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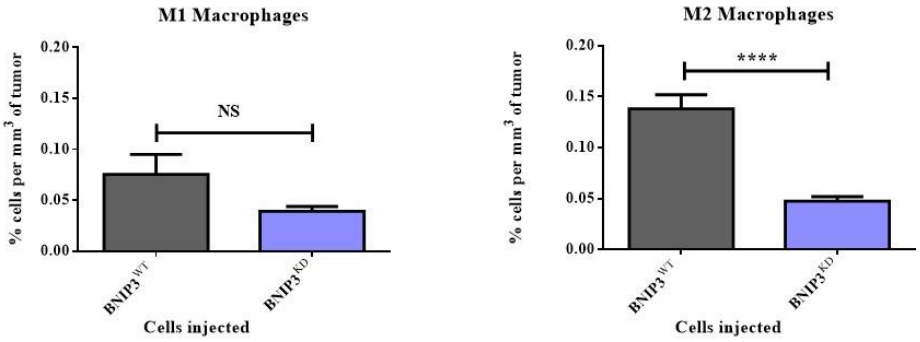

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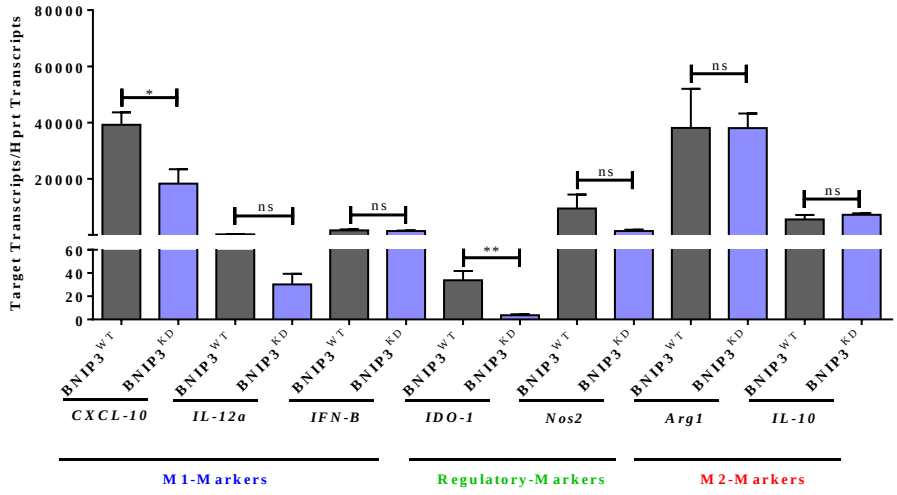

Figure 12: Sorting and characterization of the polarization status of TAMs.
(A) Analysis of the number of macrophages sorted via FACS from resected, BNIP3^{WT} and BNIP3^{KD}-B16 cells-based subcutaneous tumours. The number of sorted macrophages was normalized to tumour volume (mm^3). **(B)** Immunophenotyping on sorted TAMs, for M1 (CD86 and MHC-II) and M2 (CD206) phenotypic markers. **(C)** QRT-PCR (**Q**uantitative **R**everse **T**ranscription-**PCR**) analysis of the sorted TAMs for M1, M2 and auto-regulatory immunomodulatory markers. Data expressed as mean ± SEM and analysed with Student's *t*-test (ns = not significant; *p*-values from left to right: **p* = 0.0213; ***p* = 0.0093).

3.5. BNIP3 ablation impairs the secretion of ATP, a prototypical ICD-associated danger signal, from dying B16-F10 melanoma cells

Since the ablation of BNIP3 in B16-F10 cells, in combination with hypoxia, exerted only minor effects on the phagocytic activity and overall macrophage-activation and -polarization *in vivo* (irrespective of peritoneal or tumoural contexts), we evaluated whether BNIP3's immunomodulatory functionalities might be more evident in a cell death setup. Henceforth, we decided to study the effects of BNIP3 on chemotherapy-based ICD induction in B16-F10 melanoma cells.

To induce ICD, we treated B16-F10 melanoma cells with the *bona fide* ICD inducer MTX[143]. Both BNIP3^{WT} and BNIP3^{KD} melanoma cells treated with MTX experienced a time-dependent increase in the cleavage of caspase-3 and cell death (***Figure 13A–C***; ***see page 76***). However the overall caspase-3 cleavage wasn't significantly altered between the two conditions (***Figure 13B***; ***see page 76***). In line with this, analyses at 24 h showed no significant differences in overall cell death between BNIP3^{KD} and BNIP3^{WT} cells (***Figure 13C***; ***see page 76***). Moreover, the pan-caspases inhibitor zVAD-fmk which typically inhibits caspases-dependent apoptosis[30], significantly reduced MTX-induced killing of both BNIP3^{WT} and BNIP3^{KD} melanoma cells (***Figure 13C; see page 76***). This underscores the apoptotic nature of MTX-induced cell death in this setting. Next, we assessed the three major danger signals associated with ICD, that also serve as surrogate biomarkers of this process i.e. ecto-CALR, secreted ATP and released HMGB1[143]. Indeed, B16-F10 melanoma cells treated with MTX emitted all three danger signals (***Figure 13D–F***; ***see page 76***), in line with previous reports[144]. Interestingly, BNIP3 ablation in melanoma cells profoundly reduced (**p = 0.0035) the secretion of ATP (***Figure 13F***; ***see page 76***), without significantly affecting the emission of the other two danger signals (***Figure 13D-E***; ***see page 76***). Finally, in line with the increased ecto-CALR (***Figure 13D***; ***see page 76***) following MTX treatment, BNIP3^{WT} B16-F10 cells treated with MTX underwent rapid phagocytosis by the J774 cells (***Figure 14A***; ***see page 78***). Intriguingly, in this setting, MTX-treated BNIP3^{KD} B16-F10 cells exhibited a slight but significant reduction (*p = 0.034) in the phagocytic clearance, thereby further suggesting a pro-ICD role for BNIP3. Thus, based on the analyses of surrogate ICD

markers indicating a BNIP3-dependent ATP secretion and phagocytic clearance of dying/dead cells, BNIP3 may have a pro-ICD function in B16-F10 cells.

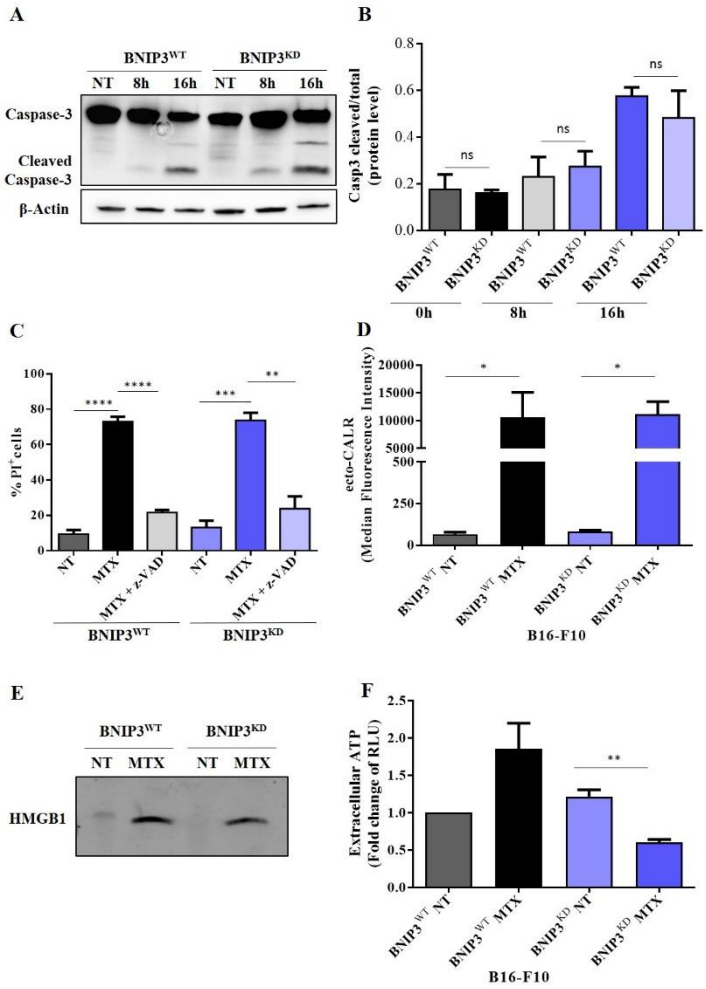

Figure 13: The secretion or surface exposure of danger signals in B16-F10 cells dying via apoptosis is partially affected by BNIP3 ablation.

(**A**) Representative immunoblot of caspase 3 cleavage in BNIP3^{WT} and BNIP3^{KD} B16-F10 cells after MTX-treatment at the indicated time points (NT: not treated). Actin was used as loading control. (**B**) Quantification of immunoblots probed with anti-Caspase 3 total and cleaved of BNIP3^{WT} and BNIP3^{KD} cells at different time-points (0 h, 8 h, 16 h). Data are expressed as mean ± SEM and analysed with Student's *t*-test (ns = not significant, $n = 3$ independent experiments) (**C**) Cell death of B16-F10 (PI+) cells after 24 h treatment with MTX and pre-/co-incubation with z-Vad-fmk measured by flow cytometry. Data are expressed as mean ± SEM and analysed with Student's *t*-test (**$p = 0.0036$, ***$p = 0.0005$, ****$p < 0.0001$, $n = 3$ biological replicates). (**D**) BNIP3^{WT} and BNIP3^{KD} B16-F10 cells were evaluated for surface exposure of CALR 16 h after MTX treatment in non-permeabilized cells via flow cytometry. Data are expressed as mean ± SEM and analysed with Mann-Whitney's *t*-test (*$p = 0.0286$, $n = 4$ independent experiments). (**E**) Representative immunoblot for HMBG1 released in concentrated conditioned medium 48 h after treatment of BNIP3^{WT} and BNIP3^{KD} B16-F10 cells with MTX. (**F**) BNIP3^{WT} and BNIP3^{KD} B16-F10 were treated with MTX and conditioned media (24 h post treatment) were analysed for the presence of ATP. Data are expressed as fold change of RLU (Relative Light Units) with respect to untreated BNIP3^{WT} (mean ± SEM) and analysed with Student's *t*-test (**$p = 0.0035$, $n = 3$ independent experiments).

3.6. The intrinsic resistance of B16-F10 to ICD-mediated anti-cancer vaccination is potentiated by BNIP3 ablation

The trends observed above with the prototypical ICD-associated danger signals, especially the BNIP3- secreted ATP link, motivated us to unravel the putative effects of BNIP3 on ICD-associated anti-cancer vaccination effect *in vivo*.

MTX-treated BNIP3^{WT} or BNIP3^{KD} B16-F10 cells were injected subcutaneously into the right flank of syngeneic immune-competent C57BL/6 mice (with PBS injected mice serving as controls). Post-vaccination, these rodents (including the PBS injected ones) were challenged with live B16-F10 cells in the opposite flank. Thereafter, protection against tumour growth at the challenge site (live B16-F10 cells) was interpreted as a sign of anti-cancer vaccination effect and expressed as tumour-free survival. To our surprise, none of the mice vaccinated with MTX treated B16-F10 cells exhibited tumour-rejecting responses (***Figure 14B***; ***see page 78***) - a sign of ICD resistance[109]. Interestingly, mice injected with MTX-treated BNIP3^{KD} B16-F10 succumbed to tumour challenge significantly faster (**$p < 0.05$) than those injected with PBS or MTX-treated BNIP3^{WT} B16-F10 cells (***Figure 14B***; ***see page 78***).

In conclusion, B16-F10 melanoma cells have an intrinsic ICD-resistant phenotype that is further potentiated by ablation of BNIP3 thereby advocating a partial pro-ICD role for BNIP3.

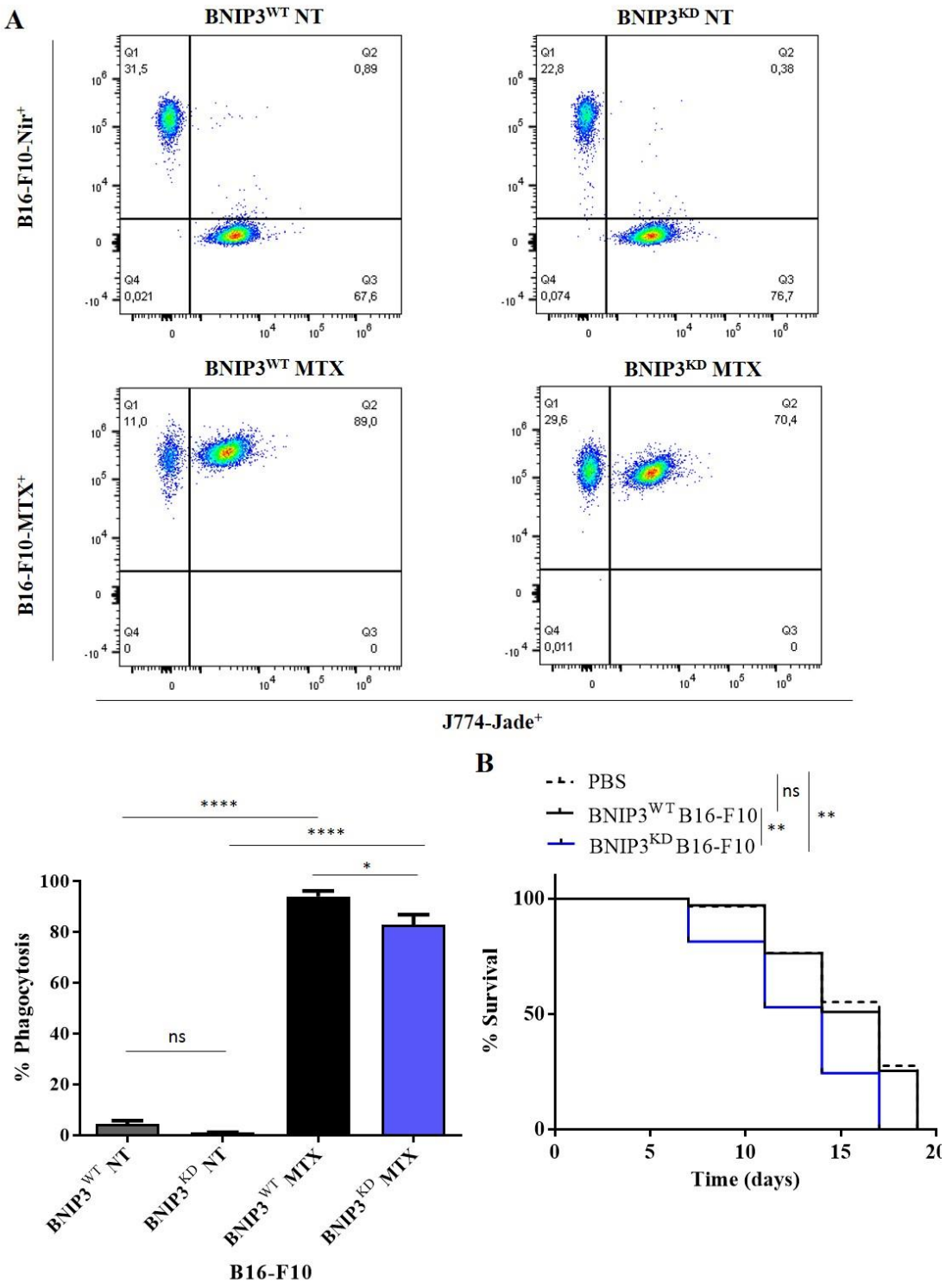

Figure 14: BNIP3 affects the phagocytic clearance of cells dying via ICD and decreases the intrinsic resistance of B16-F10 to ICD-mediated anti-cancer vaccination.

(**A**) Representative flow cytometry gates of J774-mediated phagocytosis of B16-F10 treated with MTX after 2 h co-incubation (upper panel) and relative quantification (lower panel) of B16-F10-Nir$^+$ or B16- F10-MTX$^+$ and J774-Jade$^+$ cells. Data expressed as mean ± SEM and analysed with Student's *t*-test (**p* = 0.034, *****p* < 0.0001, ns = not significant, *n* = 2 independent experiments). (**B**) Survival curve of C57BL/6 mice vaccinated with BNIP3^{WT} or BNIP3^{KD} B16-F10 cells treated with MTX or PBS control. Data are analysed with log-rank/Mantel-Cox test (***p* < 0.05, ns = not significant, *n* = 7 mice/condition).

4. Discussion

In this study, we aimed to evaluate the impact of BNIP3 ablation in melanoma cells, on the interface with immune cells on various levels particularly, phagocytosis, chemotaxis and functional polarization (all three for macrophages), as well as susceptibility to ICD. In general, we found that BNIP3 modulates melanoma cell-immune cell intersection in a highly contextual fashion and in a manner contingent upon the plasticity and immune-evasive character of the melanoma cells. For instance under normoxic conditions, despite having very low ecto-CD47, the BNIP3^{KD} B16-F10 melanoma cells were not readily phagocytosed by macrophages. Hypoxic treatment was required to accentuate these cells' phagocytic clearance. Moreover, although hypoxia elicited an increase in phagocytosis, this did not seem to be contingent upon the differential ecto-CD47 levels. We also ruled out the role of ecto-CALR (on account of its absence), a notorious 'eat me' signal known to counter-balance ecto-CD47's function[145]. These observations in melanoma cells stand in contrast to previous studies showing hypoxic or stressed cancer cells escaping from macrophage-based phagocytosis by increasing ecto-CD47[59,46,88,129]. This further highlights the complexity of the melanoma-elicited immunosuppressive paradigm. Taken together, our observations suggest that hypoxia might have a variable role in regulating phagocytic clearance depending upon the cancer-type under consideration. Nevertheless, the apparent dispensability of CD47 and the absence of ecto-CALR, despite on-going phagocytosis, may be a sign of B16-F10 melanoma cells utilizing either alternative 'don't eat me' signals or highly potent 'eat me' signals capable of circumventing ecto-CD47. Although ecto-CD47 is a very ubiquitous anti-phagocytic signal[146], yet there have been instances of other 'don't eat me' signals playing a more dominant anti-phagocytic role, like CD200 or the MHC class I-LILRB1 (**L**eukocyte **I**mmunoglobulin-**L**ike **R**eceptor subfamily **B** member **1**) signalling axes[147,148,149]. On the contrary, beyond ecto-CALR, not many 'eat me' signals have been systematically reported to counter-balance ecto-CD47[147]. These gaps in knowledge need to be addressed in near future through an explorative screening study. Finally, with respect to hypoxia's pro-phagocytic effects, whereas hypoxic environment facilitated phagocytic clearance of

B16-F10 cells *in vitro*, yet hypoxic pre-conditioning of the same cells failed to accentuate phagocytic clearance *in vivo*. At the outset, we believe this might be a result of differences in hypoxic persistence. While in the *in vitro* setting, hypoxia persisted throughout the duration of phagocytosis, in the *in vivo* setting it was systematically exerted only before the injection of the cells in the mouse peritoneum. It is conceivable that post-injection, the same level of hypoxia may not have been maintained *in vivo*, hence explaining the *in vitro* vs. *in vivo* differences in phagocytosis[150,151].

It is however of significance that we observed a failure in recruitment of macrophages by the melanoma cells in two different *in vivo* contexts i.e. a peritoneal context as well as the tumoural context. This is relevant, because these results suggest that the impact of phagocytic interface established by the BNIP3^{KD} melanoma cells may be tough to estimate, since its preceding event i.e. recruitment and/or persistence of macrophages, that have to eventually carry out phagocytic clearance, is defective to begin with. There could be a number of reasons that may explain this disparity in macrophage-recruitment; for instance: (1) changes in the TME may have impaired the infiltration and/or the survival of TAMs into the solid tumour; (2) the overall levels of chemokines secreted by melanoma cells may have failed to reach the thresholds required to recruit macrophages from the circulation into the peritoneum/tumour; (3) the mouse peritoneum is a relatively large area hence, through lavage analyses, it may not be possible to appreciate the directional re-orientation of peritoneum-resident macrophages towards the specific site of injection; and (4) similarly, it is possible that while there might be lack of TAMs in the tumour itself, yet more TAMs may exist in peri-tumoral regions where they might be establishing productive phagocytic interface with invasive melanoma cells. Nevertheless, BNIP3 proficient or deficient melanoma cells injected into the transgenic zebrafish larvae expressing mCherry^{+}macrophages (*fms:nfsB.mCherry*; to analyse localized chemotaxis close to injection site via intra-vital microscopy[137,152]), also failed to recruit the macrophages (data not showed). This suggests that BNIP3 presence does not affect either the ability of living melanoma cells to recruit these innate immune cells, or affect macrophages-polarization, at least in these two *in vivo* settings. Overall, these results do outline the extremely variable nature of the immunological interface in an *in vivo* context.

However, it would be interesting via TAMs/phagocytes depletion experiments (involving depletion via antibodies or chlodronate liposomes), to investigate the consequences of the absence of these immune cell type on the melanoma microenvironment and progression. Also relevant would be to analyse whether the phenotypes observed and caused by the loss of BNIP3 are autophagy-dependent or not, by comparison with other typical autophagy gene, such as *ATG5*. This latter comparison could be crucial because every autophagy-relevant gene, also has various non-autophagy related roles (especially in regulation of differentiation and/or cell death). And thus, differentiating these disparate roles holds the key in understanding the immunological mechanisms at play in a progressive or regressing tumour.

Remarkably, the above immune-evasive behaviour of B16-F10 cells was also visible in a therapeutic cell death setting. More specifically, despite the ability of these cells to proficiently emit prominent danger signals like ecto-CALR, secreted ATP and passively released HMGB1 after treatment with the *bona fide* ICD inducer MTX, B16-F10 cells were unable to elicit anti-cancer vaccination effect *in vivo*. In past, others and we have demonstrated that, naturally occurring or artificially-induced, defects in ecto-CALR exposure, ATP secretion or HMGB1 release can cause cancer cells to resist the pro-immunogenic effects of ICD[92,153,154,155,156]. However, B16-F10 cells proficiently emitted these danger signals thereby indicating existence of other immunoresistance pathways that need to be identified in the near future[157,158]. It also needs to be identified whether only B16-F10 melanoma cells are resistant to ICD *in vivo*, or also other known murine melanoma cellular models phenocopy this[138,159,160,161]. To address this, ICD-relevant analyses should be extended to more melanoma models, in not only transplantable but also spontaneous or autochthonous settings[159]. Such analyses could be crucial to understand whether melanoma has an intrinsic inability to undergo ICD *in vivo* or *in situ*, an interesting notion that may explain the limited success of ICD-inducing chemotherapeutics like anthracyclines against melanoma in the clinic[155].

Nevertheless, in the ICD set-up, it seemed that BNIP3 plays a partial pro-immunogenic role. More specifically, BNIP3^{KD} in B16-F10 cells caused significantly lower secretion of ATP and slightly (but significantly) impaired phagocytic clearance of dying/dead B16-F10 cells. Moreover, mice vaccinated with dead/dying BNIP3^{KD}

B16-F10 cells exhibited significantly reduced survival when compared to those vaccinated with dead/ dying BNIP3^{WT} B16-F10 cells. It is highly conceivable that the strong reduction in ATP secretion may account for this reduction in survival[130]. Of note, it may not be surprising that BNIP3 ablation affects the secretion of ATP. BNIP3 is a well-known pro-autophagic/pro-mitophagic protein[162] in melanoma cells[29] and autophagy has been widely reported to play a pivotal role in secretion of ATP following MTX[122,163,164]. Based on our results, it would be interesting to explore in the future whether mitophagy is the dominant autophagic (sub-)pathway for ATP secretion following chemotherapy. Secretion of ATP by BNIP3^{KD} B16-F10 cells under basal (untreated) conditions, was not reduced as compared to the untreated BNIP3^{WT} cells but was even slightly increased, albeit insignificantly and to an extent that is likely not biologically relevant. However, it should be mentioned that extracellular ATP secretion pathways are highly context-dependent, and thus differences may exist in ATP secretion under basal conditions as compared to specific stressed conditions. Last but not least, the partial pro-phagocytic effects of B16-F10 cells-associated BNIP3 in this cell death set-up were intriguing in the light of no apparent changes in ecto-CALR. It would be important to understand in the near future whether this indicates the presence of an alternate BNIP3-regulated 'eat me' signal.

In conclusion, we found that melanoma cell-associated BNIP3 played a highly contextual immunomodulatory role at the melanoma-immune cell interface. On one hand, in living melanoma cells BNIP3 seemed to affect immunomodulatory responses elicited by hypoxia signalling. Hence, we believe that the future studies should strive to reveal the full relevance of BNIP3 using appropriate melanoma mice models recapitulating the hypoxic tumour microenvironment. On the other hand, in response to ICD, BNIP3 accentuated ATP secretion, phagocytic clearance and the vaccination potential of the dying melanoma cells. However, we failed to fully appreciate the pro-ICD impact of BNIP3 due to the intrinsic resistance of B16-F10 cells to ICD-associated anti-cancer vaccination effect *in vivo*. It would be interesting in the future to re-examine the role of BNIP3 in cancer models susceptible to immunosurveillance and ICD. Finally, the overall role of BNIP3 in cancer growth and response to anti-melanoma therapy should be translated and validated in human melanoma.

5. Concluding remarks and future perspectives

The present study has contributed towards unravelling some interesting melanoma cell intrinsic as well as extrinsic properties associated with BNIP3. In particular, this Bcl-2 family member is able to modulate the interface between mouse melanoma cells (B16-F10) and macrophages, albeit in a context-dependent fashion. Indeed, the immunomodulatory role of melanoma cell-associated BNIP3 is differentially influenced by normoxia or hypoxia, as well as by alive or dying/dead cell status (***Figure 15***).

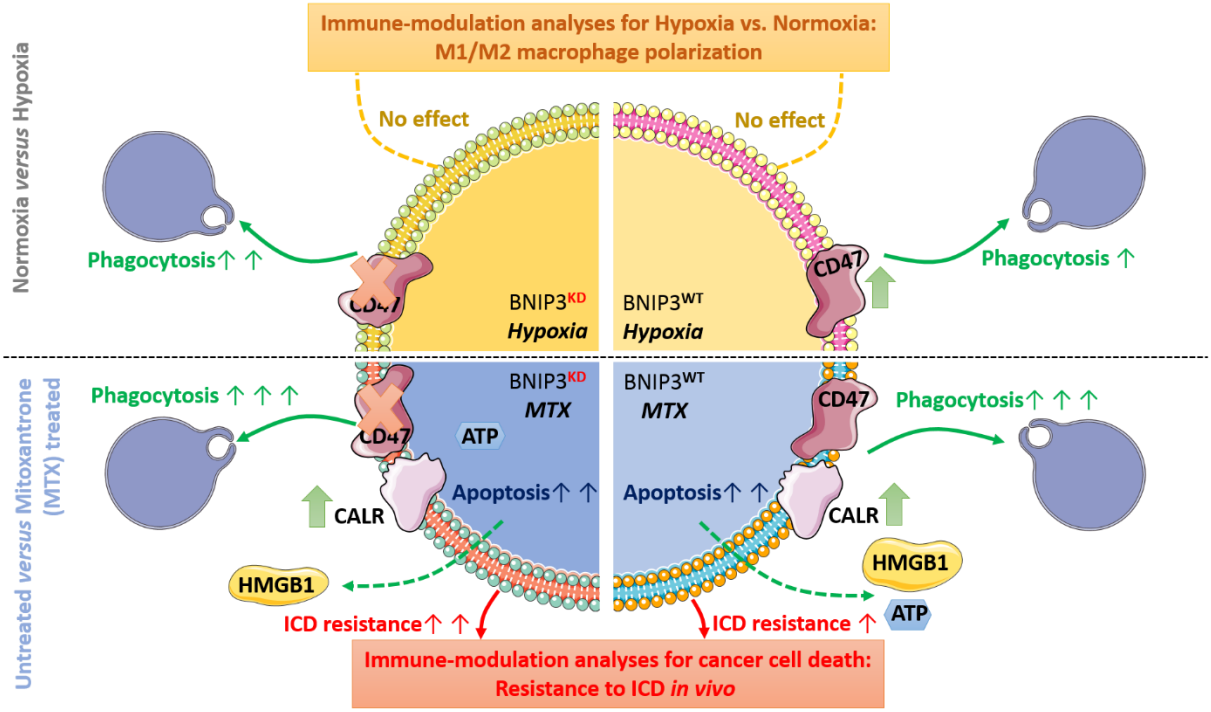

Figure 15: Concluding scheme of the research project.

BNIP3 modulates the interface between B16-F10 mouse melanoma cells and macrophages in a context-dependent manner. The immunomodulatory role of BNIP3 is differentially influenced by normoxia or hypoxia (upper panel). In particular, in both BNIP3^{WT} and BNIP3^{KD} cells, the hypoxic stimulus favours their phagocytosis by macrophages, by modulating the 'don't eat-me' signal CD47. However the BNIP3 status and/or hypoxic conditions do not have any effect on M1/M2 macrophage polarization. Additionally, BNIP3's immunomodulatory role also diverges between alive and dying/dead B16-F10 cells (lower panel). Specifically, MTX-treated cells have an intrinsic resistance to ICD-mediated anticancer vaccination, which is further potentiated by BNIP3 ablation. Indeed BNIP3^{KD} MTX-treated cells have impaired secretion of ATP, one of the major ICD-relevant danger signals.

However, there are some very interesting outstanding questions that need to be addressed in future to better decipher the immuno-modulatory role of BNIP3:

1. **Can melanoma-associated BNIP3 be considered a potential prognostic and therapeutic target?**

   Our (past as well as present) research has outlined that melanoma-associated BNIP3 has a very profound cancer cell-autonomous function directly relevant for the growth and progression of the tumour. And while various immunological functions for melanoma-associated BNIP3 were revealed in this study, yet it did not seem that these functions were collectively capable of eliciting long-term anti-melanoma immunity. On the other hand, ongoing research in our lab highlights the ability of BNIP3 –but not ATG5- to rewire the metabolism of both murine and human melanoma cells largely through the stabilization of pro-tumourigenic and immunosuppressive HIF-1a signalling both *in vitro* and *in vivo* (M. Vara-Pérez *et al*., unpublished). These studies disclose an emerging complexity of the intrinsic effects of BNIP3 in melanoma cells and their impact on the tumour microenvironment and advocate a careful analysis of BNIP3 expression in human melanoma samples at different progression stages. Preliminary results from our lab (in collaboration with Prof. J. Van den Oord) indicate that BNIP3 protein levels are invariably higher in metastatic melanoma patients. Also a TCGA (**T**umour **C**ancer **G**enome **A**tlas) analysis of *BNIP3* transcript levels in a subset of melanoma patients shows a correlation with poorer patient survival (M. Vara-Pérez *et al*., unpublished results). These preliminary observations suggest that melanoma-associated BNIP3 levels may hold a prognostic value, a point that needs further and more systematic evaluation. Furthermore, given the emerging role of TAMs in melanoma progression[165] and response to targeted therapy[166] and the ability of BNIP3 to modulate the release of critical mediators of melanoma cells-macrophages communication, more research addressing the co-presence and distribution of melanoma-associated BNIP3 and infiltrating macrophages in patient's tissue, should be performed.

Finally, considering that the recent clinical advances in melanoma immunotherapies with e.g. immune-checkpoint blockers and anti-cancer vaccines achieve efficient anti-melanoma immunity only in a subset of patients, a combinatorial treatment design to therapeutically targeting BNIP3 with a highly specific drug in melanoma cells, could be an important objective to be pursued. And once such a drug has been designed, and its biological consequence characterised, it would be then crucial to understand the combinatorial regimen with melanoma immunotherapies on the level of administration schedule, dosage, tumour immune-profile and immunotherapy-type in preclinical settings, thereby eventually paving the way for clinical translation.

2. **Does BNIP3 have a different role in melanoma cells versus immune cells?**

   As outlined above, targeted therapy directed towards BNIP3 might have promising anti-melanoma properties. However, experience with other targeted therapies has recently taught oncologists that when strategizing combinations between targeted therapy and immunotherapies, it is important to consider not only the cancer-targeting effects of the former but also the immune-targeting ones. This strategy will also be applicable to BNIP3. Unfortunately little is known about the overall effects of targeting BNIP3 in immune cells. Interestingly, a recent study found that BNIP3's pro-mitophagic functions are crucial for generation of robust long-lived "memory" NK cells[167]. Thus, therapeutic ablation of BNIP3 may curtail NK cell function. This study was primarily carried out from the vantage point of anti-viral activity of NK cells. Hence, it is not clear whether this role of BNIP3 is equally applicable to tumoural context. Moreover, every immune cells differs in their physiology, so it remains to be seen whether the above functions of BNIP3 are translatable to other immune cells, especially T cells. All of this needs to be studied, when BNIP3-targeting drugs become available. If BNIP3-targeting drugs are found to have counter-productive effects on immune cells then proper optimization of

dosage and administration schedule can be pursued to minimize such deleterious effects, while preserving the advantageous anti-cancer effects.

3. **What is the link between melanoma-associated BNIP3 and ATP secretion?**

Since the BNIP3-ICD link can also be crucial when strategizing anti-melanoma therapy, it is important to understand the exact mechanistic basis of the effects of BNIP3 on secretion of ATP during ICD. As discussed in the introduction, it has been convincingly demonstrated that autophagy is necessary for secretion of ATP following chemotherapeutic treatment. It has been showed that autophagosomes ferry the ATP towards the surface whereupon fusion with lysosomes helps in ultimate extracellular release. Previous and ongoing research in our lab has uncovered that BNIP3-ablated melanoma cells display not only defects in mitophagy but also an overall impairment in vesicular trafficking mechanisms[29] (M. Vara-Pérez *et al.*, unpublished), which are probably caused by the severe actin cytoskeleton derangement of the BNIP3-deficient cells. Thus, it is highly possible that absence of BNIP3 causes a defect in the vesicular pathway(s) of melanoma cells enabling the trafficking ATP extracellularly in response to chemotherapy, a mechanism that should be systematically investigated with high-end immunofluorescent microscopy analysis (in living cells) and co-localization studies. Finally, as mentioned in the discussion above, it is also possible that impaired mitophagy and/or changes in the bioenergetics observed in BNIP3^{KD} cells might also contribute to this phenomenon.

4. **Is there a link between BNIP3 and mitochondrial DAMPs?**

The above hypothesis suggesting a link between BNIP3 and mitochondria clearance raises another intriguing point. In the same way as postulated above, could BNIP3 be enhancing the release of mitochondrial DAMPs? It has been found that mitochondria can be a source of potent DAMPs especially mtDNA (**mit**ochondrial **DNA**) and N-formylpeptides[168]. Therefore, it is possible that in BNIP3^{KD} cells, lack of mitophagy creates a larger pool of mitochondria that can

ultimately translate into larger bioavailability of mitochondrial DAMPs. Thus, if this hypothesis is correct, BNIP3-ablated tumours might have a richer source of mitochondrial DAMPs that can be released extracellularly upon necrotic cell death (such that DAMPs like mtDNA or N-formylpeptides can initiate danger signalling by binding to their cognate receptors like TLR9 or FPR1 (**F**ormyl **P**eptide **R**eceptor **1**), respectively, on immune cells) or intracellularly in the cytosol through mitochondrial permeabilization during apoptosis (leading to danger signalling via cytosolic DNA-sensing pathways)[168,169]. These intriguing possibilities need to be tested in the near future.

6. Materials and Methods

6.1. Cell culture

B16-F10 were cultured at 37° C, either under 5% CO_2 and 20% O_2 (normoxia) or 5% CO_2 and 1.5% O_2 (hypoxia), in RPMI medium (Sigma, R8758) supplemented with 10% FBS (**F**oetal **B**ovine **S**erum), 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin. Generation of shRNA stable clones was performed as previously described[29]. Murine macrophages J774 were kindly provided by S. van den Brule (UCL) and cultured in DMEM medium and supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

6.2. Cell death induction and quantification

MTX was purchased from Sigma-Aldrich (M6545) and used at a concentration of 8 μM to induce immunogenic cell death. Apoptosis induction was counteracted by pre-treating for 4 h with the pan-caspases inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (zVAD-fmk, 50 μM) purchased from Bachem (N1560). To estimate the amount of cell death, supernatant and cells were collected with TrypLE Express and resuspended in PBS containing propidium iodide (PI) (Sigma, P4170). Samples were subsequently acquired via Attune Flow Cytometer (Life Technologies) and data analysis was performed via FlowJo_V10™ software (Tree Star, Ashland, OR, USA).

6.3. Flow cytometry-based detection of cell surface CD47 and CALR

B16-F10 cells were washed with PBS, detached with TrypLE Express (ThermoFisher, 12604–21), centrifuged and washed once with ice-cold FC (**F**low **C**ytometer) buffer (2% BSA, 1% FBS in PBS). For the detection of CD47, cells were incubated with FITC-conjugated anti-CD47 (ThermoFisher, 11-0471-82) in FC buffer at 4° C for 45 min. For the detection of ecto-CALR instead, cells were incubated with anti-CALR (Abcam, Ab92516) at 4° C, for 40 min, in FC buffer followed by Alexa Fluor647 Anti-Mouse IgG (ThermoFisher, A-27040) at 4° C, for 40 min. Then the cells were resuspended in FC

buffer including 1 µmol/L Sytox Green (Life Technologies, S7020). Due to the high autofluorescence of MTX, the background fluorescent measured in the unstained sample was subtracted from the median fluorescent intensity of the relative stained samples. Cells were acquired with Attune Flow Cytometer and data analysis was performed via FlowJo_V10™ software.

6.4.*In vitro* phagocytosis assay

B16-F10 and J774 were detached with TrypLE Express and labelled respectively with CellVue®Nir780 (Affymetrix eBioscience, 88–0875) and CellVue®Jade (Affymetrix eBioscience, 88–0876). J774 were kept in serum-free medium for 4 h and subsequently co-incubated with B16-F10 at a 1:5 ratio (Macrophages : Cancer Cells) for 24 h, under both normoxic and hypoxic conditions. Alternatively B16-F10 were treated with MTX and co-incubated for 2 h with J774 at a 5:1 ratio (Macrophages : Cancer Cells). The autofluorescence of MTX was used as label of cancer cells. Cells were harvested using TrypLE Express and acquired with Attune Flow Cytometer. Data analysis was performed via FlowJo_V10™ software.

6.5.Immunoblotting

Cells were lysed in a buffer containing 100 mM Hepes 7.4, 10% sucrose, 1% Triton x100, 2.5 mM EDTA, 5 mM DTT, 1 mM PMSF, 2 µg/ml pepstatin, and 2 µg/ml leupeptin. For BNIP3 extraction instead, cells were lysed with a modified Laemmli buffer[29]. Proteins were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane and analysed by immunoblotting. Primary antibodies detecting BNIP3 (Biokè, 3769S), total (Biokè, 9662S) and cleaved (Biokè, 9661S) caspase 3, HMGB1 (Genetex, GTX12029) and β-actin (Sigma) were further detected with the appropriate horse radish peroxidase-conjugated secondary antibody (Thermo Scientific), whose reaction was in-turn incited with Pierce ECL Western Blotting Substrate (ThermoFisher, 32106). Immunoblotting for concentrated conditioned media-associated HMBG1 was carried out as reported before[137]. Quantification of the images was performed via ImageJ.

6.6. Antibody array

B16-F10 proficient and deficient for BNIP3 were kept under normoxia or hypoxia and after 24 h the supernatant was collected, centrifuged and processed according to manufacturer's instructions (R&D systems, ARY015). The membranes were scanned using the Bio-Rad Chemidoc Imager (Bio-Rad Laboratories N.V.3, Winninglaan, Temse, Belgium) while ImageJ was used for the quantification.

6.7. ATP assay

The cells were treated as indicated. Extracellular ATP was measured in the conditioned medium (2% FBS) via an ATP Bioluminescent assay kit (Sigma) based on luciferin-luciferase conversion, following manufacturer's instructions. Bioluminescence was assessed by optical top reading via FlexStation 3 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

6.8. Tumour models and prophylactic mouse vaccination

Mouse experiments were performed at KU Leuven (Leuven, Belgium) in the designated animal facilities and in accordance with the institutional and national guidelines and regulations. Animals were purchased from the internal stock of the animal facility at KU Leuven (Leuven, Belgium) or from external companies. Animal procedures were approved by the Institutional Animal Care and Research Advisory Committee (KU Leuven) (P139/2014).

To assess subcutaneous tumour growth, B16-F10 melanoma cells ($BNIP3^{WT}$ and $BNIP3^{KD}$) were harvested and single-cell suspensions of 150.000 (in 100 µL PBS) were subcutaneously injected into the right flank of immunocompetent syngeneic (C57BL/6) female mice (6–9 weeks old). Tumour growth was followed closely, while tumour volumes were measured three times per week with a calliper (using the formula V = p 3[d23 D]/6, where "d" and "D" are respectively the minor and the major tumour axis). At the moment of scarification, tumours were collected for further analysis/experiments.

The prophylactic vaccination was performed in immunocompetent syngeneic (C57/Bl6) female mice (6–9 weeks old), via subcutaneous injection of 200 µL PBS with dying B16-F10 cells (3 x 10^6 cells in 200 µL PBS) or 100 µL of PBS only into the right flank, two-times in a time frame of 2-weeks. After 7 days from the second injection, mice were challenged with untreated BNIP3^{WT} B16-F10 cells (vector-free) into the left flank (5 x 10^5 cells in 100 µL PBS) and tumour growth was monitored for 20 days.

6.9. Flow Sorting of TAMs (Tumour Associated Macrophages)

Melanoma-bearing mice were sacrificed by cervical dislocation and tumours resected as before[170]. In brief, tumours were minced in RPMI medium containing 0.1% collagenase type I and 0.2% dispase type II and incubated in the same solution for 30 min at 37 °C. The digested tissues were further dissolved mechanically. The myeloid cell population in the tumour single cell suspension, was enriched by magnetic isolation using CD11b-conjugated magnetic beads and subsequently, stained for the pan-macrophage marker F4/80.

6.10. Immunonophenotyping of TAMs via FACS

In order to assess the polarization status of the TAMs, part of the sorted macrophages were stained for M1 or M2-markers, specifically for CD86 and MHC-II or for CD206. In detail, sorted TAMs were spun down, re-suspended in PBS containing 2% FBS and the respective antibodies (fluorophore conjugated from Affimetrix e-Bioscience) were added. Incubation was performed for 40 min, on ice and in the dark. After the staining, the cells were spun down and fixed in 1% PFA, for 15 min, at RT (**R**oom **T**emperature). Cells were kept at 4 °C until acquisition via FACS.

6.11. QRT-PCR of sorted TAMs

QRT-PCR (**Q**uantitative **R**everse **T**ranscription-**P**olymerase **C**hain **R**eaction) was performed as before[170]. In brief: part of TAMs sorted were centrifuged as described previously[170], collected in RLT Buffer and kept at -80 °C until RNA extraction and

cDNA synthesis. At the moment of the extraction, 70% EtOH (**E**thanol) was added to each thawed sample that was then transferred into an RNeasy MinElute spin column. Samples were centrifuged for 30 sec at 10.000 rpm and the procedure was carried out according to manufacturer's instructions (RNeasy Micro Kit 74004-Qiagen). The RNA content was measured by mean of a Nanodrop 2000. For the cDNA synthesis, the Superscript III First Strand (Invitrogen, 18080-05) was used and the procedure was conducted according to manufacturer's instructions. Commercially available probes (Integrated DNA technologies) were used for the investigated genes. The PCR-primer IDs are listed in ***Table 2*** (***see page 93***).

6.12. *In vivo* phagocytosis assay

C57BL/6 female mice (6–9 weeks old) were injected intra-peritoneum with PBS containing 3 x 10^6 B16-F10 cells (BNIP3^{WT} or BNIP3^{KD}) pHrodo-labelled (Life Technologies, P36600). The peritoneal cells were collected 24 h post-injection via peritoneal lavage with Ca^{2+}- and Mg^{2+}-free PBS. The cells were transferred to an ultra-low attachment V-bottom plate and stained with the fixable Live/Dead Yellow stain (Invitrogen). After blocking the F_c receptor (CD16/32, eBioscience, 16-0161-82), cells were stained for F4/80-eFluor780 (eBioscience, 47-4801-80), CD11b-eFluor450 (eBioscience, 48-0112-82), MHC-II-FITC (eBioscience, 11- 5321-81), CD86-APC (eBioscience, 17-0862-81), CD206-PeCy7 (eBioscience, 25-2061-80) in FC buffer. Samples were acquired on a Gallios™ flow cytometer and the data analysis was performed via FlowJo_V10™ software.

6.13. Statistical analysis

The statistical analyses were performed via GraphPad Prism 6 (Graphpad Software, San Diego, CA, USA) and are indicate in the respective figure legends. Grubbs' test was used to exclude outliers, where applicable.

Table 2. IDT's identifiers for the Taqman assays (primers and probe) used for the QRT-PCR

Gene	Assay ID (company: IDT)
HPRT	Mm.PT.58.32092191
Arg1	Mm.PT.58.8651372
Nos2	Mm.PT.56a41998785
CXCL-10	Mm.PT.58.43575827
IDO-1	Mm.PT.5829540170
IL-10	Mm.PT.58.13531087
IL-12	Mm.PT. 58.13818295

7. List of figures and tables

Figures

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8. Conflicts of interest

The authors have no conflict of interest to declare.

9. Funding

This research was supported by C16/15/073 grant of the KU Leuven, FWO grant G0584.12 and STK F/2014/222 grant to P.A. Erminia Romano is supported by a fellowship of the Kom Op tegen Kanker (Stand up to Cancer), the Flemish cancer society. Abhishek D. Garg and Hannelie Korf were supported by the FWO Postdoctoral Fellowship. Abhishek D. Garg was additionally supported by the POR award funds from KU Leuven. Nicole Rufo is supported by European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant (Agreement no. 642295).

10. Contribution by other scientists

The data presented in this thesis are the main results of my PhD project, carried out at the CDRT (**C**ell **D**eath **R**esearch and **T**herapy) laboratory, under the supervision of Prof. Patrizia Agostinis. I have personally contributed to the majority of the experimental part presented in this manuscript, with the exception of the *in vitro* section about ICD. However, since this research required additional expertise in the field of macrophages and TAMs, we started two collaborations with the laboratories of Prof. Massimiliano Mazzone and Prof. Chantal Mathieu. In particular, contribution to this work was given by: Pawel Bieniasz-Krzywiec, Sarah Trusso Cafarello, Jens Serneels (from Prof. M. Mazzone's lab) and Hannelie Korf (from Prof. C. Mathieu's lab). Also colleagues from the CDRT team contributed to this project, namely: Nicole Rufo, Mónica Vara Pérez, Dr. Abhishek D. Garg, Dr. Hannelore Maes, Dr. Aleksandra M. Dudek-Perić, Dr. Shaun Martin and Ester Vanmol.

11. Professional Career

Fellowships

Kom Op tegen Kanker (Stand up to Cancer), the Flemish cancer society.

Curriculum Vitae

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EDUCATION

Graduate Education

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PhD thesis project being performed at the Cell Death Research and Therapy Laboratory, Department of Cellular and Molecular Medicine, Faculty of Medicine, KU Leuven, Belgium under the supervision of **Prof. Patrizia Agostinis.**

- February 2017– active participation in IUAP meeting, Ghent, Belgium (poster);
- September 2016 – active participation in Cell Symposia: 100 years of Phagocytosis, Sicily, Italy (poster);
- September 2016 – active participation in LKI Symposium, KU Leuven, Belgium (**oral presentation** and poster);
- June 2016 – active participation in Oncoforum, KU Leuven, Belgium

(poster and **poster prize won**);

- June 2015 – active participation in Oncoforum, KU Leuven, Belgium (poster);
- February 2015 – active participation in IUAP meeting, Ghent, Belgium (oral presentation and poster);
- October 2014 – active participation in ECDO conference, Crete, Greece (poster);
- June 2014 – active participation in Oncoforum, Leuven, Belgium (poster);
- January 2014 – active participation in IUAP meeting, Ghent, Belgium (poster);

November 2012-April 2013

24-weeks stage at "INRA (Institut National de la Recherche Agronomique) Centre" (Jouy en Josas, France), for having **awarded a grant** within the "Unipharma-Graduates 8" Project –"Leonardo Da Vinci" Program.

Undergraduate Education

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Master Degree in "Biology", awarded at the university "Università degli Studi di Napoli Federico II" (Naples, Italy) with a score of 110/110 cum laude;

- Master Thesis Project: "CD180: a signalling mediator in B-CLL" was performed at "University of Westminster" (London, UK) within the "Program LLP-Erasmus" (**awarded grant, year 2009-2010**).

October 2008-November 2011

Master studies carried out at the university "Università degli Studi di Napoli Federico II" (Naples, Italy).

October 2008

Bachelor Degree in "General and Applied Biology", awarded at the university "Università degli Studi di Napoli Federico II" (Naples, Italy) with a score of 110/110 cum laude.

October 2005-October 2008

Bachelor studies carried out at the university "Università degli Studi di Napoli Federico II" (Naples, Italy).

12. Publications

1. Garg, A., Galluzzi, L., Apetoh, L., Baert, T., Birge, R., Bravo-San Pedro, J., Breckpot, K., Brough, D., Chaurio, R., Cirone, M., Coosemans, A., Coulie, P., De Ruysscher, D., Dini, L., de Witte, P., Dudek-Peric, A., Faggioni, A., Fucikova, J., Gaipl, U., Golab, J., Gougeon, M.,Hamblin, M., Hemminki, A., Herrmann, M., Hodge, J., Kepp, O., Kroemer, G., Krysko, D., Land, W., Madeo, F., Manfredi, A., Mattarollo, S., Maueroder, C., Merendino, N., Multhoff, G., Pabst, T., Ricci, J., Riganti, C., **Romano, E.**, Rufo, N., Smyth, M., Sonnemann, J., Spisek, R.,Stagg, J., Vacchelli, E., Vandenabeele, P., Vandenberk, L., Van den Eynde, B., Van Gool, S., Velotti, F., Zitvogel, L., Agostinis, P. (2015). Molecular and Translational Classifications of DAMPs in Immunogenic Cell Death. *Frontiers in Immunology, 6*, art.nr. e588.

2. Garg, A., Dudek-Peric, A., **Romano, E.**, Agostinis, P. (2015). Immunogenic cell death. *The International Journal of Developmental Biology, 59* (1-3), art.nr. 10.1387/ijdb.150061pa, 131-140.

3. Garg, A., Maes, H., **Romano, E.**, Agostinis, P. (2015). Autophagy, a major adaptation pathway shaping cancer cell death and anticancer immunity responses following photodynamic therapy. *Photochemical & Photobiological Sciences, 14* (8), art.nr. 10.1039/c4pp00466c, 1410-24.

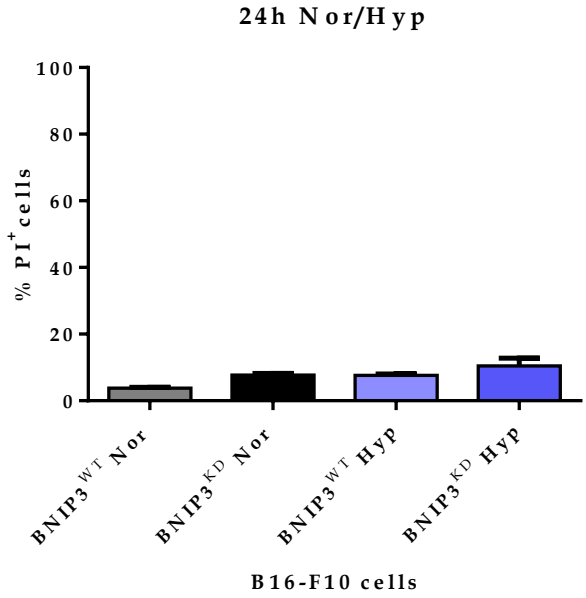
4. AD Garg, **E Romano**, N Rufo and P Agostinis. Immunogenic versus tolerogenic phagocytosis during anticancer therapy: mechanisms and clinical translation (2016). *Cell Death and Differentiation*; 23(6):938-51. doi: 10.1038/cdd.2016.5

5. EV-TRACK Consortium, Van Deun J, Mestdagh P, Agostinis P, Akay Ö, Anand S, Anckaert J, Martinez ZA, Baetens T, Beghein E, Bertier L, Berx G, Boere J, Boukouris S, Bremer M, Buschmann D, Byrd JB, Casert C, Cheng L, Cmoch A, Daveloose D, De Smedt E, Demirsoy S, Depoorter V, Dhondt B, Driedonks TA, Dudek A, Elsharawy A, Floris I, Foers AD, Gärtner K, Garg AD, Geeurickx E,

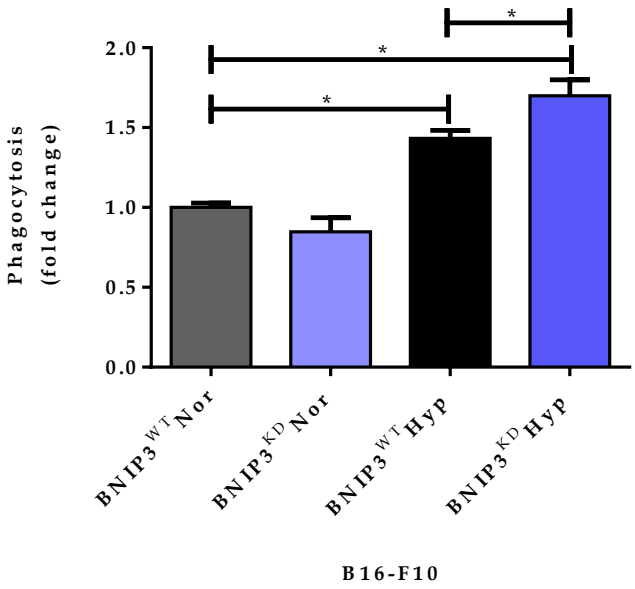
Gettemans J, Ghazavi F, Giebel B, Kormelink TG, Hancock G, Helsmoortel H, Hill AF, Hyenne V Kalra H, Kim D, Kowal J, Kraemer S, Leidinger P, Leonelli C, Liang Y, Lippens L, Liu S, Lo Cicero A, Martin S, Mathivanan S, Mathiyalagan P, Matusek T, Milani G, Monguió-Tortajada M, Mus LM, Muth DC, Németh A, Nolte-'t Hoen EN, O'Driscoll L, Palmulli R, Pfaffl MW, Primdal-Bengtson B, **Romano E**, Rousseau Q, Sahoo S, Sampaio N, Samuel M, Scicluna B, Soen B, Steels A, Swinnen JV, Takatalo M, Thaminy S, Théry C, Tulkens J, Van Audenhove I, van der Grein S, Van Goethem A, van Herwijnen MJ, Van Niel G, Van Roy N, Van Vliet AR, Vandamme N, Vanhauwaert S, Vergauwen G, Verweij F, Wallaert A, Wauben M, Witwer KW, Zonneveld MI, De Wever O, Vandesompele J, Hendrix A (2017). EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nat Methods*. 2017 Feb 28;14(3):228-232. doi: 10.1038/nmeth.4185.

6. **Erminia Romano**, Nicole Rufo, Hannelie Korf, Chantal Mathieu, Abhishek D. Garg, Patrizia Agostinis. BNIP3 modulates the interface between B16-F10 melanoma cells and immune cells**.** *Oncotarget*. 2018; 9:17631-17644. doi.org/10.18632/oncotarget.24815

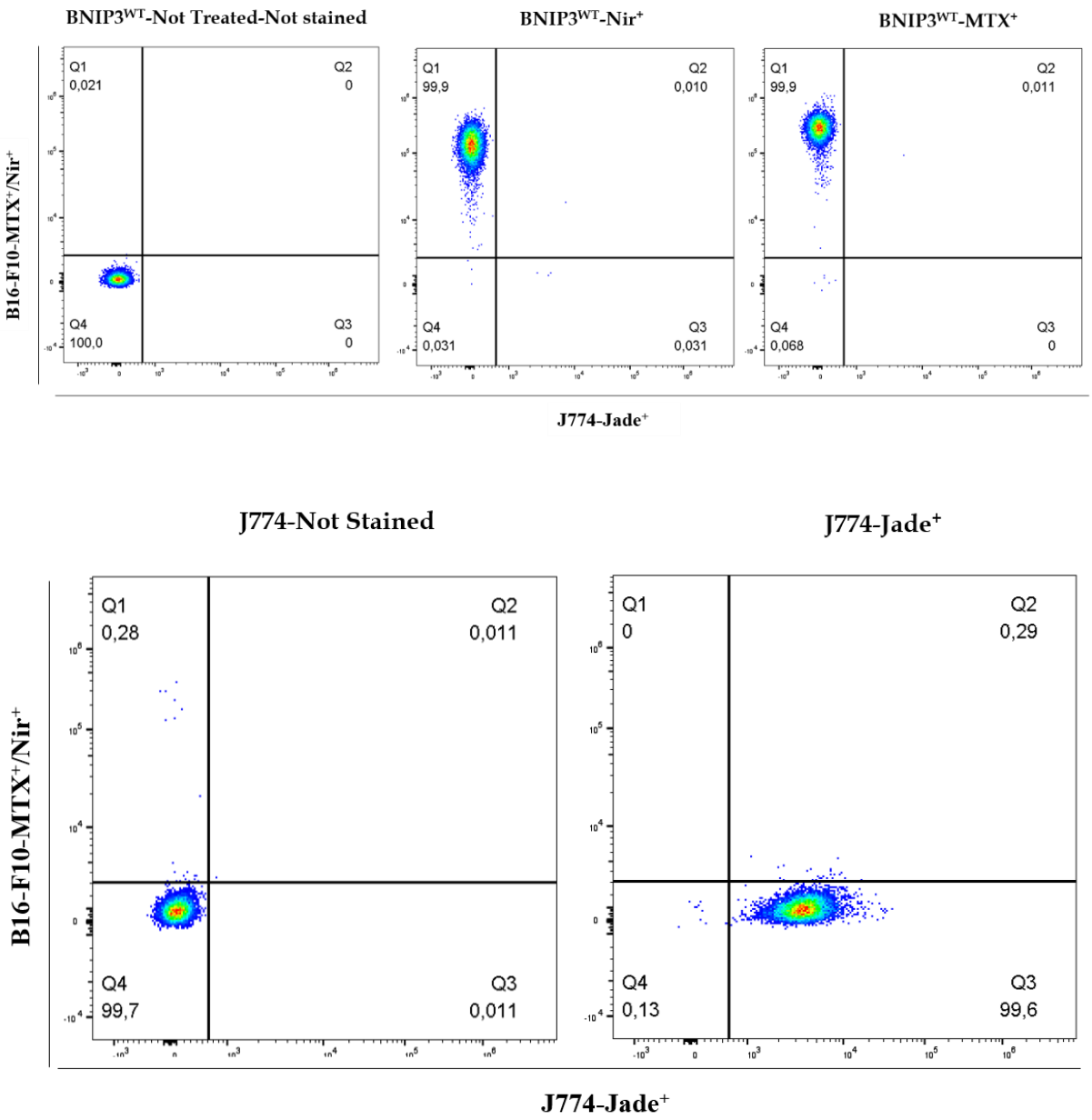
13. Addendum



Addendum for Figure 6C:
Cell death of B16-F10 (PI^+) cells, kept 24 h under normoxia (Nor) and hypoxia (Hyp), measured by flow cytometry. Data are expressed as mean ± SEM and analysed with Student's t-test.



Addendum for Figure 6C:
Relative quantification of J774-mediated phagocytosis of B16-F10 kept 24 h under normoxia (Nor) and hypoxia (Hyp), measured by flow cytometry. Data are expressed as mean ± SEM and analysed with Student's t-test.



Addendum for Figure 14(A): BNIP3 affects the phagocytic clearance of cells dying via ICD and decreases the intrinsic resistance of B16-F10 to ICD-mediated anti-cancer vaccination.

Representative dot plots for the single cell populations, stained (with Nir or Jade) or unstained, treated or untreated (with MTX).

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