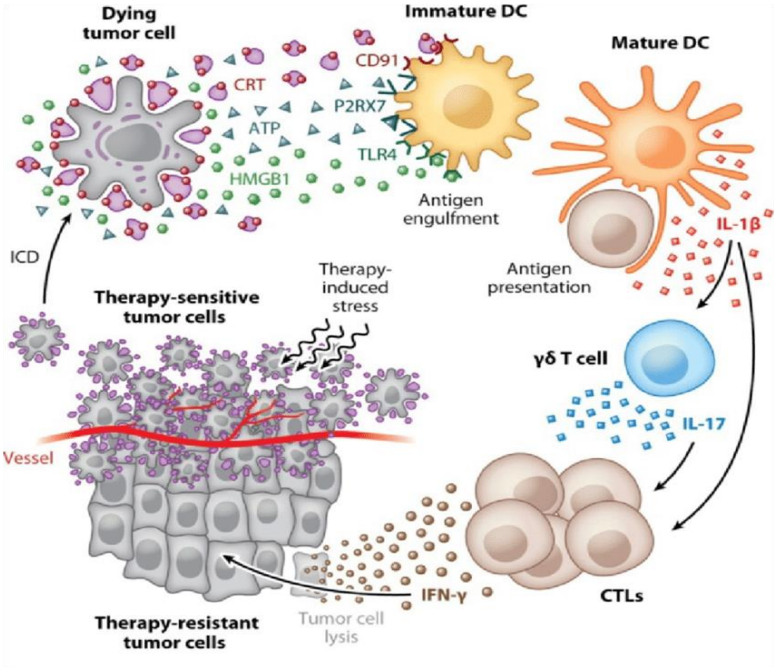
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXV CYCLE

Maria Marotta

Novel immunogenic therapeutic combinations in anaplastic thyroid carcinoma



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Novel immunogenic therapeutic combinations in anaplastic thyroid carcinoma

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LIST OF ABBREVIATIONS

TC: Thyroid carcinoma
GLOBOCAN: Global Cancer Observatory
WDTC: Well differentiated thyroid carcinoma
PDTC: Poorly differentiated thyroid carcinoma
ATC: Anaplastic thyroid carcinoma
PTC: Papillary thyroid carcinoma
FTC: Follicular thyroid carcinoma
MTC: Medullary thyroid carcinoma
TSH: Thyroid stimulating hormone
NGS: Next Generation Sequencing
MAPK: Mitogen-activated protein kinase
PI3K: Phosphatidylinositol-3 kinase
AKT: Protein kinase B
TERT: Telomerase reverse transcriptase
BRAF: v-raf murine sarcoma viral oncogene homolog B1
TCGA: The Cancer Genome Atlas
NIS: Sodium iodide symporter
RAS: Rat sarcoma virus
GDNF: glial line-derived neurotrophic factor
RET: Rearranged during transfection
RAI: Radioactive iodine therapy
EIF1AX: Eukaryotic translation initiation factor 1 A, X-linked
PAX8-PPARγ: Paired box 8/peroxisome proliferator activated receptor γ
mTOR: Mammalian target of rapamycin
TP53: Tumor protein P53
LOF: Loss of function
PTEN: Phosphatase and tensin homolog
CTNNB1: Catenin beta-1
TME: Tumor microenvironment
TAMs: Tumor-Associated Macrophages
DCs: Dendritic Cells
TAMCs: Tumor-Associated Mast Cells
TANs: Tumor-Associated Neutrophils
NKs: Natural Killer Cells
CTLs: CD8+ Cytotoxic T Cells
Treg: Regulatory T lymphocyte cells
CSF-1: Colony stimulating factor-1
CCL2: C-C motif chemokine ligand 2
MCs: Mast cells
IL8: Interleukin 8
EMT: Epithelial-to-mesenchymal transition
SLUG: Zinc finger protein SNAI2.
NLR: Neutrophil to lymphocyte ratio
GM-CSF: Granulocyte macrophage colony-stimulating factor

CD56: Neural cell adhesion molecule
CD16: Low affinity immunoglobulin gamma Fc region receptor III-B
CXCR1: C-X-C Motif Chemokine Receptor 1
CXCR2: C-X-C Motif Chemokine Receptor 2
CD3: Cluster of differentiation 3
CD28: Cluster of differentiation 28
ICs: Immune checkpoints
PD-1: Programmed cell death-1
PD-L1: Programmed cell death-ligand 1
PD-L2: Programmed cell death-ligand 2
ICIs: Immune checkpoint inhibitors
MKIs: Multi kinase inhibitors
RAIR: RAI refractory
MEK: Mitogen-activated protein kinase kinase
TKIs: Tyrosine multi-kinase inhibitors
FDA: Food and Drug administration
CTLA-4: Cytotoxic T-Lymphocyte Associated Protein 4
TNM: Tumor, Node, Metastasis
PFS: Progression free survival
ICD: Induction of Immunogenic cell death
ER: Endoplasmic reticulum
DAMPs: Dangers associated molecular patterns
APCs: Antigen-presenting cells
TAAs: Tumor-associated antigens
TNAs: Tumor neoantigens
CRT: calreticulin
ATP: Adenosine Triphosphate
HMGB1: high mobility group box 1
ANXA1: annexin A1
CXCL10: cytokine like C-X-C motif chemokine ligand 10
CD91: Cluster of differentiation 91
LRP1: Low density lipoprotein receptor-related protein 1
eIF2α: Eukaryotic Translation Initiation Factor 2 subunit alpha
P2RY2: Purinergic Receptor P2Y2
P2RX7: Purinergic Receptor P2X 7
IFN I: Interferon type I
CXCR3: C-X-C Motif Chemokine Receptor 3
AGER: advanced glycosylation end-product specific receptor
TLR4: Tool like receptor 4
EGFR: epidermal growth factor receptor
RTK: Tyrosine kinase receptor
HDAC: histone deacetylase
PDT: photodynamic therapy
SHP2: Src homology region 2 (SH2)-containing protein tyrosine phosphatase 2
EGF: epidermal growth factor

FBS: fetal bovine serum
sPD-L1: Soluble PD-L1
FACS: Fluorescence activated cell sorting
Ab: Antibody
BrdU: Bromodeoxyuridine
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ECL: Enhanced chemiluminescence
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SD: Standard deviation
IgG4: Immunoglobulin G_4
PTPN11: protein tyrosine phosphatase non-receptor type 11
SH2: Src homology 2
HCC: Hepatocellular carcinoma
NSCLC: Non-small cell lung cancer
ICB: Immune checkpoints Blockade

ABSTRACT

Anaplastic thyroid carcinoma (ATC) is a rare, undifferentiated, aggressive and therapy-resistant tumor of the thyroid follicular epithelium. Conventional chemo/radiotherapy, and targeted therapies directed against components of the Ras/MAPK pathway - a driver oncogenic cascade in thyroid carcinoma (TC) - have been used, but they have shown limited efficacy.

Immunogenic cell death (ICD) is a form of apoptosis induced by specific stressors, able to evoke tumor regression through a durable antitumor immune response. Single compounds or combination therapies have been shown to induce ICD in distinct tumor types. Here, we wish to identify novel therapies capable of inducing ICD in ATC. To this aim, we used novel drug combinations against recently identified molecular targets to induce ICD in human ATC.

One of the potential targets is represented by the immune checkpoint (IC) Programmed cell death-1 (PD-1). We found that ATC cells express both PD-1 and its ligands, PD-L1/2. The PD-1 intrinsic circuit promoted ATC cell proliferation, migration and tumorigenicity in immunocompromised mice. Accordingly, Nivolumab, a PD-1 neutralizing antibody, blocked these effects. PD-1-mediated activities in ATC cells required the SHP2 tyrosine phosphatase, that, upon membrane recruitment by PD-1, can activate Ras, thus triggering the MAPK pathway. Indeed, SHP099, a SHP2 inhibitor, could block PD-1-mediated mitogenic and migratory activities of ATC cells.

Another potential target, previously identified by us in ATC, is represented by interleukin 8 (IL8), an inflammatory chemoattractant cytokine that, by binding its receptors CXCR1/CXCR2, induces proliferation, survival, stemness, motility, tumor formation and immunosuppressive features of ATC cells. The blockade of the IL8 circuits with Reparixin, a non-competitive CXCR1/2 inhibitor, repressed IL8-mediated activities in ATC cells. Furthermore, IL8 potentiated the immunosuppressive properties of TC cells by increasing the expression of IC molecules on their surface.

Since PD-1/SHP2 and IL8/CXCR1/2 circuits play a critical role in the maintenance of aggressive ATC features, we tried different combinations of Reparixin, Nivolumab and SHP099, in order to test whether the simultaneous blockade of these circuits could kill murine and human ATC cells in an immunogenic fashion. We found that SHP099, in combination with Nivolumab or with Reparixin, and Nivolumab in combination with Reparixin, exerted cytotoxic/cytostatic effects on both murine and human ATC cell lines. Importantly the formers combinations were more efficient than the latter in inducing ICD hallmarks, including eIF2α phosphorylation, calreticulin (CRT) exposure on cell surface, ANXA1, ATP and HMGB-1 increase. Thus, SHP099, in combination with Nivolumab or Reparixin, will be tested *in vivo* in syngeneic mice models in order to assess their ability to induce tumor regression and to elicit durable immunity against ATC.

1. INTRODUCTION

1.1 Thyroid Cancer

Thyroid is an endocrine gland situated in the lower part of the neck, anteriorly to the trachea. It consists of two lobes joined together by the isthmus. The main functional and structural unit of the thyroid gland is the thyroid follicle, which is made up of a single layer of epithelial cells, also known as thyroid follicular cells. These cells are responsible of iodine trapping from circulation and thyroid hormone production. In between thyroid follicular cells there are the neuroendocrine C cells, also known as parafollicular cells, involved instead in the secretion of the polypeptide hormone calcitonin.

Thyroid cancer (TC) is the most prevalent endocrine neoplasia with an increasing incidence over the past three decades, as reported by epidemiological data from world cancers registers (1). The latest Global Cancer Observatory (GLOBOCAN) survey from 2020 reports that TC is responsible for 586,000 cancer cases worldwide (2), moreover, if recent trends are maintained, TC may become the fourth most common cancer by 2030 in the United States (3).

Multiple factors have been associated with the risk of developing TC, including female gender, age, obesity, radioiodine exposure, genetic alterations, history of goiter, and family history of thyroid disease (4).

TC can arise from either follicular or parafollicular cells of the thyroid gland. Medullary Thyroid carcinoma (MTC) originates from parafollicular cells. TC arising from follicular cells are classified based on their histological and clinical features in well-differentiated (WDTC), poorly differentiated (PDTC) and undifferentiated/anaplastic (ATC) histotypes. WDTCs comprise papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) (**Figure 1**). PTC and FTC are the most frequent forms (90%) of TC, distinguished from the other forms by a relatively favorable prognosis. They are characterized by typical histological features, and by the maintenance of some differentiating markers such as the ability to capture iodine, synthesize thyroglobulin and respond to thyroid stimulating hormone (TSH). Most of patients experiencing WDTC (80-85%) have a good response to conventional therapies, including surgery, radioiodine treatment and TSH suppressive therapy (5); however a consistent percentage of patients, about 15-20%, displays recurrence or persistence of neoplasia, generally associated with resistance to radioiodine therapy (6). PDTCs and ATCs are the most aggressive forms of TC, accounting about 1-5% of TC, often associated with a poor prognosis due to aggressive behavior and short median time of survival, caused by the lack of successful treatment. In the most accepted theory of follicular cell carcinogenesis, different molecular alterations have been associated with specific steps, leading progression from well differentiated to undifferentiated forms of TC (7) (**Figure 1**). According to this model, it has been observed the existence of differentiated or poorly differentiated areas within ATC samples, suggesting that there is a progressive dedifferentiation process of WDTC that leads to ATC (8).

Thanks to the advent of new genome sequencing techniques [i.e., Next Generation Sequencing (NGS)], in the last three decades a huge number of

studies have characterized the genetic and epigenetic aberrations involved in TC development (9–12). Indeed, more than 90% of mutations underlying TC pathogenesis have been identified, such as mutations that provide a selective growth advantage thus promoting cancer development (13) . The main molecular alterations that lead to TC initiation and progression involve mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/AKT signaling pathways. Several additional mutations in other cellular pathways, such as p53, Wnt/β-catenin or mutations in TERT promoter, are involved in TC progression to PDTCs and ATCs (**Figure 1**).

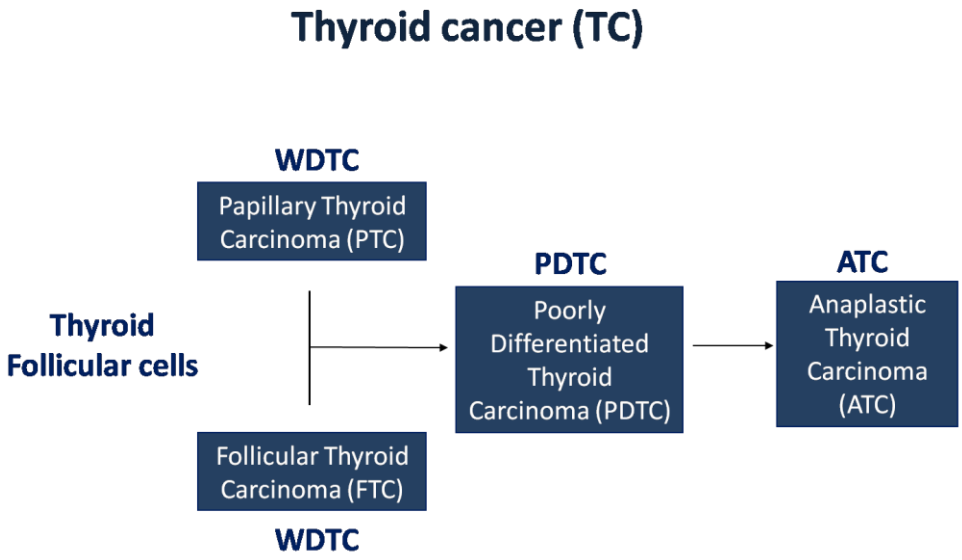

Figure 1. Thyroid carcinomas (TCs) derived from thyroid follicular cells. TCs are the most common tumors of the endocrine system and can originate either from follicular cells or parafollicular C cells. From follicular cells derive well-differentiated forms (WDTC), such as papillary carcinoma (PTC) and follicular carcinoma (FTC), poorly differentiated carcinomas (PDC) and undifferentiated forms, such as anaplastic carcinoma (ATC).

1.2 Genetic landscape of follicular cell-derived thyroid carcinoma

1.2.1 Papillary thyroid carcinoma (PTC)

PTCs are the most common subtype of TCs, mostly characterized by good prognosis in terms of long term-survival (90%) (13,14). PTCs comprise various histological subtypes: classical (CV), follicular (FV), tall cell (TCV) variant and other uncommon PTC variants.

Typical driver mutations found in nearly 70% of PTCs include point mutations of *BRAF* or *Ras* and *RET* rearrangements (15) occurring in a mutually exclusive modality. Genetic alterations of these molecules result in a constitutive activation of MAPK signaling leading to PTC initiation (15).

The most frequent mutation in PTCs involves BRAF, that encodes for a serine/threonine protein kinase belonging to the RAF family. A recent analysis from The Cancer Genome Atlas (TCGA), reported that 74.6% of PTCs carry BRAF mutations, of which 61.7% were V600E substitutions (15). Other genetic

alterations have been identified in this gene; however, the majority of classical variant PTCs harbors the BRAFV600E mutant (15). The BRAFV600E driver mutation triggers a constitutive activation of MAPK signaling that, in turn, causes loss of differentiation, tumor progression and apoptosis inhibition (16). In particular, a down regulation in genes required for thyroid hormone biosynthesis, including thyroglobulin and the sodium iodide symporter (NIS), have been described in PTCs harboring this type of mutant (17–19). Moreover, this mutation has been described by several studies to be associated with aggressive features, such as lymph node metastases, invasion, and recurrence (20,21).

The RAS family of small GTP-binding proteins includes the four different but related proteins H-Ras, N-Ras, K-Ras4A, and K-Ras4B, that act upstream of BRAF, and signal through the MAPK and PI3K-AKT pathways, controlling cell growth, differentiation, and survival. Ras proto-oncogenes were found mutated in PTCs (22) with an overall prevalence of 10-15%, especially in the follicular variant (23). Point mutations in codon 61 of N-Ras and, to a lesser extent, of H-Ras are the most frequently observed (24). Conversely to BRAF mutations, Ras mutations define a less aggressive subsets of PTC tumors.

The *RET* gene encodes for a single-pass transmembrane receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor (GDNF) family. Chromosomal rearrangements of N-terminal domains of unrelated gene(s) with the C-terminal kinase domain of RET result in the production of chimeric forms of the receptor (RET/PTC) that are constitutively active in thyroid cells. RET/PTC rearrangements have been identified in sporadic and radiation-associated PTCs (25). Among the twelve rearrangements reported (26), the most common are RET/PTC1 (resulting from the fusion between the tyrosine kinase domain of *RET* and *CCDC6*) and RET/PTC3 (resulting from the fusion of the tyrosine kinase domain of *RET* with *NCOA4*) (27). According to the recent report of TCGA, in a series of 484 PTCs belonging to different ethnic groups, only 6.8% presented RET rearrangements (15). In general, patients that have RET/PTC1 and RET/PTC3 have a favorable prognosis due to their ability to respond to radioactive iodine (RAI) therapy (28).

1.2.2 Follicular thyroid carcinoma (FTC)

Follicular thyroid carcinomas represent the second most common type of TC, accounting for 10-15% of WDTC cases. Point mutations in the *Ras* gene family (K, -H and -N) are found in 20-50% of FTCs (29), thus representing the most common lesion involved in FTC pathogenesis (30). The prognostic value of Ras mutations in FTC is still unclear; a previous study has demonstrated that Ras mutations are negative prognostic markers (31), whereas recent studies didn't identify Ras mutations as predictors of disease-specific mortality (32).

PAX8-PPARγ (paired box 8/peroxisome proliferator activated receptor γ) rearrangement is the second common mutation found in FTC cases, ranging from 12% (33) to 53% (34). PAX8 belongs to paired box family of transcription factors, and it is involved in thyroid development, thyroid progenitor survival

and in the expression of thyroid-specific genes (35) . On the other hand, PPARγ, a member of the nuclear receptor family of transcription factors, is required for adipocyte differentiation and lipid metabolism (36) and it is considered a tumor suppressor gene (37). The fusion protein PAX8-PPAR*γ* acts as dominant negative regulator of wild type PPAR*γ* (38). Importantly, it has been found that PAX8-PPAR*γ* rearrangement and Ras mutations are mutually exclusive (39).
Finally, telomerase reverse transcriptase (TERT) point mutations have been reported in nearly 15% of FTC cases and are associated with bad prognostic and clinical features (40).

1.2.3 Poorly differentiated thyroid carcinoma (PDTC)

PDTCs represent rare and aggressive thyroid malignancies, accounting for approximately 5% of all TCs, are associated with a mean survival of 3.2 years after diagnosis. According to progressive dedifferentiation model of TC tumorigenesis, PDTCs share some of pathognomonic WDTC molecular alterations, and in addition they gain many other genetic alterations leading to dedifferentiation.
In terms of biological features and aggressiveness, PDTCs are located in between WDTCs and ATCs. Indeed, these tumors are characterized by a higher mitotic index, infiltrative growth and vascular invasion, with respect to WDTCs (41). BRAFV600E mutations occurs in 33% of PDTCs, while Ras mutation are found in 28% of cases (11). BRAF and Ras mutations are mutually exclusive and denote diverse clinical behavior: BRAF-mutated PDTCs generally have a high rate of nodal metastases and show low expression of thyroid-specific genes related to radioiodine avidity; by contrast, Ras mutated counterparts show a high rate of distant metastases, and no evidence in alteration of genes involved in radioiodine avidity (11,42).
Additional mutations that contribute to the pathogenesis of PDTCs are clonal point mutations in TERT promoter, ranging from 33% (43) to 40% (11) of PDTCs. Intriguingly, TERT promoter mutations are correlated with a higher risk of distant metastases and mortality (11).The eukaryotic translation initiation factor 1 A, X-linked, EIF1AX, was found mutated in 10% of PDTCs cases and its mutation is associated with worse clinical criteria (11). This protein is a component of the preinitiation complex (PIC), which is involved in the protein translation. EIFIAX mutations were associated with larger tumors and predicted shorter survival in PDTCs (11). Moreover, EIF1AX mutations often occurred with Ras mutations (42).
The PI3K/AKT/mTOR pathway regulates different cellular processes such as cell metabolism, vitality, and motility. Mutations in components of this cascade have been found in 11% of PDTCs (11).
The *TP53* tumor suppressor gene encodes for a nuclear protein involved in cell-cycle arrest, senescence, and/or apoptosis in response to different stimuli. Mutations that cause a loss of function (LOF) in this gene have been extensively considered a hallmark of advanced forms of thyroid neoplasia (44,45). Previous studies reported that inactivation of TP53 was found in 8% of PDTC cohort cases

(11), whereas recent study report that no one of 21 patients affected by PDTCs was positive for TP53 alterations (43).
Chromosomal rearrangements that are common in WDTCs, such as RET/PTC and PAX8-PPARγ, have been found in 14% of PDTCs (43).

1.2.4 Anaplastic thyroid carcinoma (ATCs)

ATCs, also known as undifferentiated TCs, although representing 1-2% of TC cases, are the most aggressive type of follicular cell-derived TCs, and unfortunately associate to a patients' mean survival of only six months after diagnosis. ATCs can originate from preexisting WDTCs or they can arise *de novo* (46,47). Conversely to other forms of follicular cell-derived TCs, ATCs lose many features of normal follicular cells, like iodine uptake, thyroglobulin synthesis, as well as TSH dependence, rendering this type of tumors refractory to conventional TC therapies (42). From a clinical point of view, ATCs is characterized by a high rate of growth, metastasis and a rapidly fatal clinical outcome. With respect to PDTCs, ATCs have a higher tumor mutation burden (11). The most common driver mutation present in ATCs is BRAFV600E, present in 45% of ATCs, whereas mutations in N-, K-, or H-Ras occurre in 24% of ATCs (11,43) and they are mutually exclusive with BRAF mutations and RTK gene rearrangements. TERT promoter point mutations and TP53 LOF mutation are the most common alterations in ATCs (11,43). Importantly, since TP53 is mutated at high frequency in ATCs, it is reported to be pathognomonic for these tumors (11). EIF1AX was found mutated in 9% of ATCs cases and it is strongly associated with Ras mutations (48). Differently to PDTCs, the PTEN/PI3K/Akt pathway was found mutated with a high prevalence in ATCs (39%). A huge number of ATCs (65%) display mutations in CTNNB1 gene, that encodes for β-catenin. This protein is normally involved in the maintenance of cell adhesion, while, when mutated, is sequestered in the nucleus where it acts as transcriptional factor, favoring the expression of genes promoting tumor growth (49). Additional alterations found in ATCs with high prevalence (36%) are represented by genes encoding components of the SWI/SNF chromatin remodeling complex (11). Moreover, genes involved in epigenetic modifications, in cell cycle regulation as well as in DNA mismatch repair, were found mutated in ATCs, whereas no chromosomal aberrations were found (11).

1.3 Tumor microenvironment (TME): a focus on immune landscape of TC

In the context of tumor initiation and progression, a critical role is played by tumor microenvironment. The crosstalk between cancer cells and the different cellular and non-cellular players of this environment might drastically influence the destiny of cancer progression. In this scenario, immune cells infiltrating the tumor bed have been extensively studied (50). To date, the main immune cell populations identified in TC microenvironment are Tumor-Associated Macrophages (TAMs); Dendritic Cells (DCs); Tumor-Associated Mast Cells (TAMCs); Tumor-Associated Neutrophils (TANs); Natural Killer Cells (NKs); $CD8^+$ Cytotoxic T Cells (CTLs); $CD4^+$ T helper cells including Regulatory T

lymphocyte (Treg cells) **(Figure 2)**. Non-cellular components of TME are represented by many cytokines involved in tumor initiation and progression, such as interleukin 8 (IL8) (51).

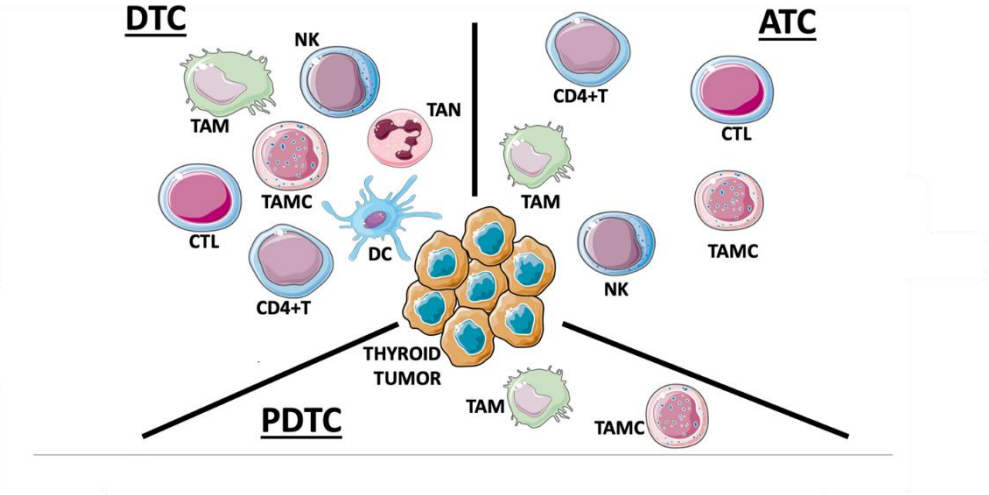

Figure 2. Representative immune cells in tumor microenvironment (TME) of thyroid tumor histotypes. TME of DTCs is the most studied. ATCs have been also shown to be infiltrated by cells and mediators of immune system. PDTCs display TAMs and TAMCs in their TME: DTC, differentiated thyroid cancer; ATC, anaplastic thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; NK, natural killer; TAM, tumor-associated macrophage; TAN, tumor-associated neutrophil; TAMC, tumor-associated mast cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; $CD4^{+}T$, $CD4^{+}$ T helper cell. Modified by Menicali *et al.*, 2021 (5).

1.3.1 Roles of immune cells in thyroid TME

Tumor-Associated Macrophages (TAMs) represent the most abundant immune cells in TME of TC. A huge number of studies reported that TAM infiltration in TCs correlates with clinic-pathological features of this malignancies. Specifically, it has been shown that in PTCs, TAMs positively correlated with increased tumor size, lymph node metastasis and reduced survival (52,53). Moreover, it has been described that production of IL8 by TAMs in human PTCs, promotes invasiveness of TC cell lines *in vitro* (53). Intriguingly, PTCs harboring BRAFV600E mutations show an increased in TAM infiltration (54). A murine model of PTCs was useful to demonstrate that TAM infiltration is due to Colony stimulating factor-1 (CSF-1) and C-C motif chemokine ligand 2 (CCL2) production by TC cells (54).

Also in PDTCs, TAMs show a fundamental role in conferring an aggressive phenotype to these tumors. Indeed, increased TAM infiltration strongly correlates to histological grade, extra-thyroid invasiveness, and decreased survival in PDTCs (55).

In comparison to WDTCs and PDTCs, ATCs display the highest TAM density in tumor bed, representing more than 50% of infiltrating immune cells (56). Moreover, it has been shown that the presence of these cells in TME of ATCs

directly correlates with poorer overall survival and with worst clinical outcome (56).

Dendritic cells (DCs) that infiltrate TCs show an immature phenotype characterized by low expression of costimulatory molecules, high expression of inhibitory molecules, and an immunosuppressive secretory phenotype (57). Since these cells show an impaired antigen presenting ability, they are unable to completely activate T cells. Very few studies are available regarding the role of DCs in TC context indicating that: i) DCs cells are recruited to PTC niche because cancer cells express various molecules with chemotactic activity that enhance their recruitment (58); ii) PTCs display an increased DCs infiltration with respect to both normal thyroid tissue and FTCs (58,59); iii) PTCs harboring BRAFV600E mutations exhibit an increased level of DC infiltration in comparison to PTCs that do not carry this mutation (60); iv) conversely to WDTCs, DCs infiltration is not very common in PDTCs and ATCs (61).

Tumor-Associated Mast Cells (TAMCs) role in TCs was firstly characterized by Melillo *and coll*. They showed that TAMC infiltration was higher in PTCs sample compared to normal thyroid tissues and it correlated with tumor extra thyroid extension (62). Moreover, they demonstrated that in PDTCs and ATCs samples, TAMC density correlated with tumor invasiveness (63). Finally, they showed that TAMCs play a pro-tumorigenic role in TC context: TAMCs produced the proinflammatory IL8 cytokine that induced epithelial-to-mesenchymal transition (EMT) and the acquisition of stemness features in TC cells (63). In FTCs, high TAMC infiltrate was found increased with respect to adenoma, and correlated with extracapsular extension (64).

Many evidence suggest a critical role of Tumor-Associated Neutrophils (TANs) in cancer biology (65,66). Despite in several tumor context the high peripheral blood neutrophil to lymphocyte ratio (NLR) was found correlated to larger tumor size and high risk of recurrence (67), in TC NLR was not associated with a dismal disease-free survival or higher risk to metastasis (68). Galdiero *et al.* found that a high TAN density correlated with larger TC size. Moreover, they showed that TC cell lines, by secreting IL8 and GM-CSF, promoted neutrophil recruitment and survival, respectively (69).

Natural Killer Cells (NKs) are distinguished in two major subtypes: $CD56^{dim}$ $CD16^{+}$ NK cells, that show a higher cytotoxic activity and $CD56^{bright}$ $CD16^{-/low}$, that are more efficient in cytokine production (70). In PTCs, NK density was higher compared to goiters and healthy thyroids (71). In patients affected by ATC, the levels of circulating NKs were significantly higher in comparison with healthy subjects, with an enrichment in $CD56^{bright}$ $CD16^{-/low}$ NKs subtype, less prone to cytotoxic activity, suggesting that ATC mediates suppression of NKs within tumor bed (72).

The T lymphocyte compartment infiltrating tumors includes both $CD8^{+}$ and $CD4^{+}$ cells (73). It is well known that, among the others, the two main population affecting cancer progression are the $CD8^{+}$ cytotoxic lymphocytes, with the potential to kill cancer cells, and the regulatory subset of $CD4^{+}$ T lymphocytes ($CD4^{+}CD25^{+}FoxP3^{+}$ Tregs), which instead contributes to dampen anti-tumor

immunity (74,75). In well differentiated TCs, $CD8^+$ T cells infiltration is associated with good prognostic features (76). In more aggressive forms of TC, there is an enrichment of $CD8^+$ cells negative for granzyme B, indicating a state of anergy of these cells (77). Various reports indicate that there is a direct correlation between tumor Treg infiltration and aggressive traits of TCs. In PTCs, Tregs frequency correlated with lymph node metastasis and recurrence (78,79). It has also been described that, in PTCs carrying BRAFV600E mutation, low intra-tumoral $CD8^+/Foxp3^+$ ratio was associated with an increased expression of immunosuppressive molecules, such as programmed death-ligand 1 (PD-L1) (80). Recently, it has also been demonstrated an involvement of Tregs cells in the immunosuppressive behavior of ATCs, due to their expression of the immune checkpoints PD-1 and PD-L1 (81).

1.4 Role of IL8 in TME of TC

The contribution of TME to cancer progression is not only associated to the presence of specific subsets of immune cells and to their phenotype, but also to the soluble components of TME, which can directly impact on cancer cell functions or indirectly contribute to cancer progression by affecting the phenotypic characteristics of TME infiltrating cells (82).

Interleukin 8 (IL8) is a proinflammatory cytokine expressed by several cancer types that contributes to cancer aggressive features (83). Consistent data revealed that IL8 is an active player also in TC context, representing an important marker of aggressiveness in this neoplasia (51,84).

Visciano *and coll.* demonstrated that TAMCs, by producing IL8, could activate the epithelial-to-mesenchymal transition (EMT) and increase the stemness features of TC cells (63). Liotti *et al.* found that IL8 was not only produced by tumor stroma, but also by TC cells, and that TC cells overexpress also the IL8 receptors, CXCR1/R2 (85). By characterizing the role of IL8 in TC scenario, they found that, regardless of its source, IL8 contributes to various TC aggressive features, sustaining cell proliferation, survival, motility, inducing EMT and stemness properties (63,85).

Consistently, IL8 serum levels are associated with TC stage (84), and IL8 staining intensity of human TC samples is correlated with the presence of lymph-nodal metastasis (85). Consistently with the key role of IL8 in TC aggressiveness, Liotti *et al.* demonstrated that Reparixin, an allosteric dual inhibitor of the IL8 receptors CXCR1/R2, affected various IL8 mediated biological functions in TC, but did not exert effects on normal thyroid compartment (86). Specifically, Reparixin reverted IL8 mediated cell proliferation, survival, EMT, stemness and tumorigenic potential of TC cells (86). Moreover, Reparixin potentiated both *in vitro* and *in vivo* the cytotoxic activity of classical chemotherapeutic agents (i.e., Doxorubicin, Docetaxel) (86). These data, taken together, suggest that IL8 circuit blockade by using Reparixin (or other IL8 circuit inhibitors), both alone or in combination with chemotherapy, might represent a new strategy for aggressive forms of TC that do not respond to conventional therapeutic regimen (86).

1.5 Immune escape of TCs driven by immune checkpoints (ICs)

The immune system is able to recognize and eliminate precancerous/cancerous cells before they become harmful for the host. Unfortunately, despite an extensive immune surveillance, cancer cells, in certain circumstances, succeed in circumventing immune system attacks through different mechanisms (87), by a phenomenon called cancer immuno-editing, that results in tumor immune escape. Within tumor bed, cancer cells modulate the host immune cells by recruiting immunosuppressive cells, by reducing tumor immunogenicity, or by exploiting immunosuppressive mechanisms (88).

The upregulation, by cancer cells, of membrane-associated immune-inhibitory molecules is the one of most relevant tumor immune escape mechanism. The immune checkpoints (ICs), such as programmed cell death-1 (PD-1), and programmed cell death-ligand 1 (PD-L1) and 2 (PD-L2), normally involved in the maintenance of self-tolerance and in switching off T cell-mediated immune response, are among the most important inhibitory molecules expressed within tumor stroma responsible for the inhibition of the anti-tumor immune response (88).

PD-1 is a membrane receptor expressed on the surface of activated T and B lymphocytes and by NK cells, that, by binding its ligands PD-L1 or PD-L2, inhibits the effector function of CTLs, thus attenuating the immune response (88). It has been shown that tumors express high levels of PD-L1 or PD-L2 and exploit this mechanism as an immune escape strategy (87). Different studies have investigated the expression of ICs and their relevance in TC (89–91). These studies confirmed the expression of PD-L1/L2 on TC epithelial cells of both WDTCs and ATCs and showed that there is a heavy PD-1^{+} T cell infiltrate, thus indicating that TCs are “hot tumors”, and they could be capable of respond to immunotherapy since their expression of ICs (5). Several studies reported that there is a positive correlation between PD-L1 expression and BRAFV600E (15,80,92). Moreover, a recent meta-analysis highlighted that positive PD-L1 expression is significantly associated with poor survival in TC patients and that increased PD-L1 expression has a significant positive association with disease recurrence (93). Finally, the expression of PD-L1 was significantly associated with increased tumor size and multifocality in PDTCs (94). To date, no consistent data about the correlation of PD-L2 expression levels and clinical features of TCs patients are reported.

An emerging and promising approach treatment for cancer is based on the use of immune checkpoint inhibitors (ICIs). ICIs basically are neutralizing monoclonal antibodies against ICs, which are used in clinic in order to block the co-inhibitory signals, thus re-activating a prolonged anti-tumor immune response (91). The expression of ICs in TCs sets the stage for the employment of immunotherapeutic approaches also for the advanced forms of these tumors. Nowadays various clinical trials are testing the employments of ICIs alone or in combination with other agents, such as multi kinase inhibitors (MKIs), in advanced form of TCs (91). It has been demonstrated that pembrolizumab (anti-PD-1) alone enabled the complete surgical resection of a BRAF-mutated ATCs.

Furthermore, pembrolizumab showed to be an effective salvage therapy added to tyrosine kinase inhibitors (TKIs) in a subset of patients with ATC (91). Although produced on few patients, these reports demonstrated that ICI treatment was well tolerated and may represent an effective treatment with robust sustained responses (91).

1.6. Treatment strategies for TCs

American Thyroid Association Management Guidelines indicate that adult patients affected by WDTCs are treated with a multidisciplinary approach involving surgeons, endocrinologists, medical oncologists, and nuclear medicine radiologists.

When tumor is resectable (low-risk, differentiated thyroid cancer with a tumor size smaller than 4 cm) surgery is still the initial treatment of choice (95). After surgical resection, WDTC management includes radioactive iodine (RAI) ablation and thyroid-stimulating hormone (TSH) suppression in case of high-risk groups (95). However, in 10% of WDTCs, surgical resection and RAI ablation fail, and patients experience recurrence and distant metastasis. These tumors, together with PDTCs and ATCs, are RAI refractory (RAIR) and are considered "advanced thyroid cancers". These malignancies represent a therapeutic challenge since although patients received a multimodal therapeutic approach (standard therapies and cytotoxic chemotherapy), treatment efficacy has been very limited (96).

In the specific case of an ATC, if the tumor is resectable, first line treatment includes radiotherapy followed by a combination of chemotherapies (cocktails of taxanes, anthracyclines, and platinum-based cytotoxic agents) (47). However, no clear evidence for improvement in quality of life or survival with these approaches in ATCs has been found (47). Overall, ATC response rate to standard systemic therapies is suboptimal, usually <15% (97).

Since somatic mutations are well characterized in TCs, MAPK signaling inhibitors have been introduced in the management of advanced thyroid cancer patients (47). Unfortunately, the occurrence of resistance to these pharmacologic approaches is common (98). This clinic challenge together with the development of RAIR in few WDTCs, in most PDTCs and in ATCs, favored studies investigating new therapeutic approaches or combinatorial strategies designed to overcome primary and/or secondary drug resistance or RAI refractoriness.

It has been found that MAPK signaling inhibitors exert cytostatic effects on TC cells but they can also achieve RAI re-sensitization (47,98). In particular, in a phase 2 clinical trial (NCT02152995), treatment with trametinib, a second generation MEK inhibitor, followed by RAI therapy, enhanced RAI uptake/efficacy in a subset of Ras and BRAF mutated tumors. Sorafenib and Lenvatinib, two TKIs, have been approved by the Food and Drug administration (FDA) in the management of patients with RAIR progressive WDTCs (98). Different other TKIs are currently investigated in RAIR tumors in phase 2 and 3 trials, in which lenvatinib is the most studied (NCT03573960, NCT03506048, NCT02702388, and NCT03139747 among others) (47). Several trials continue

to investigate the optimization of RAI treatment either as monotherapy (NCT00415233) or in combination with TKIs (NCT03506048, NCT02393690), cytotoxic agents (NCT03387943), and BRAF + MEK inhibitors (NCT03244956).

New combinatorial strategies aim also to increase the rate of ATC response to drugs. Tubulin-binding compounds, e.g. paclitaxel, fosbretabulin, and TKIs/MKIs have been utilized in phase 1/2 studies with highly variable response rates (47). In a phase 2, open-label trial, in 16 patients with ATC subjected to prior radiation and/or surgery and/or systemic therapy, the safety and efficacy of combination therapy with dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) was evaluated. The clinical study reported an overall response rate of 69%, including 1 complete response (97). Concordant results were also obtained in a basket trial that had enrolled patients with non-melanoma cancers carrying BRAFV600E disease-causing variants. Among the 7 ATC patients who had tried prior systemic therapies, one patient had a complete response, and another patient had a partial response, and these responses were sustained for more than 12 months (99). Thus, FDA approved BRAF/MEK inhibitor therapy combination for the management of BRAFV600E-positive ATCs.

Also, PI3K/AKT/mTOR pathway, activated in about 30-35% of ATCs, represents a target for therapy (47). Several phase 2 clinical studies evaluated everolimus, a mTOR inhibitor, in patients with locally advanced or metastatic ATCs, showing an increase in progression free survival (PFS) (47). Clinical trials, testing other novel therapies for ATCs, include ALK inhibitors (ceritinib, NCT02289144), other selective mTOR inhibitors (sapanisertib, NCT02244463), and efatutazone (PPARγ agonist, NCT02152137).

Since TCs display immune cell infiltrate with an enrichment in the expression of CTLA-4 and PD-L1 positive cells, also immunotherapy continues to be actively investigated (91). Examples of the immune checkpoint inhibitors (ICIs) under studies in ATCs include atezolizumab (anti-PD-L1) (NCT03181100), nivolumab (anti-PD-1) (NCT03246958), ipilimumab (anti-CTLA-4) (NCT03246958), pembrolizumab (NCT03211117) (anti-PD-L1), durvalumab (NCT03122496) (anti-PD-L1), tremelimumab (anti-CTLA-4) (NCT03122496), and spartalizumab (anti-PD-1) (NCT02404441).

The partial results of a phase II of the study have been published regarding the spartalizumab efficacy in patients with ATCs. Forty-two patients were enrolled, reaching an ORR of 19% and a PFS of 1.7 months (100). Another clinical trial evaluated the clinical efficacy of Nivolumab and Ipilimumab as single institution in a cohort of 13 patients with metastatic ATCs. This trial reported an objective response rate of 16% (2/13), and a one-year survival rate of 38% (5/13) (101).

Even though up to now multidisciplinary approaches have been used, ATC management remains very challenging. Effective treatment strategies are highly needed.

1.7 Induction of immunogenic cell death (ICD) as a novel therapeutic strategy for ATC

In an imaginary perfect scenario, we should have the possibility to manage pharmacologic approaches able to provide a durable and complete response to cancer. A possibility to get close to this goal is to design a strategy that educate our immune system to recognize and kill cancer cells overcoming the immune escape mechanisms activated in cancer cells. This could be reached by increasing the "visibility" of cancer cells and in the meantime by improving the antitumor immune response.

In this context, several research groups are investigating pharmacologic strategies able to kill cancer cells favoring the release of several cell components with immunogenic, antigenic, and/or proinflammatory potential. This kind of induced cell death is to date defined as "Immunogenic Cell Death - ICD" (102). For decades a unique paradigm differentiated between homeostatic (i.e., self and non-antigenic) and pathogen-induced (i.e., non-self and antigenic) forms of cell death, being the first one typically non inflammatory, while the second one an inflammation-inducing cell death. Today, it is clear that also cell death provoked by endogenous forms of damage could induce inflammation, becoming immunogenic (102).

ICD is characterized by the induction of endoplasmic reticulum (ER) stress and is accompanied by the exposure, active production, or release of numerous dangers associated molecular patterns (DAMPs) and inflammatory cytokines. DAMPs, in the context of an inflammatory microenvironment, make cancer cells 'visible' to immune system, in particular to dendritic cells (DCs). In this way, DCs engulf dying tumor cells and initiate a strong antitumor immune response, able eventually to also block tumor growth. Thus, ICD inducers can drive an inflammatory response and trigger the activation of cytotoxic T lymphocyte (CTL)-driven adaptive immunity coupled with the establishment of long-term immunological memory (102) (**Figure 5**).

Antigenicity and adjuvanticity are two key factors for ICD induction. Antigenicity is determined by the expression and presentation of antigens that are not subjected to central tolerance, implying that the host contains naïve T cell clones that can recognize such antigens (103). Neoplastic cells express a panel of antigenic epitopes for which naïve T cell clones are generally available, thus displaying sufficient antigenicity to drive immune response (103). Adjuvanticity is provided by the release or exposure of DAMPs, that are necessary to trigger inflammation and for the recruitment and maturation of antigen-presenting cells (APCs), i.e., DCs (103).

Cancer cells can initiate an antitumor immunity by expressing tumor-associated antigens (TAAs). TAAs are not unique to tumor tissue but are also expressed by healthy or immune-privileged tissues (103). The recognition of these antigens by central tolerance is leaky, and peripheral tolerance can be overcome in the context of robust adjuvanticity (103). Furthermore, TAAs have been shown to induce anticancer immunity, especially in the setting of therapeutic anticancer vaccination (103).

Tumor-related antigenicity is correlated to the elevated mutational rate that typically accompanies neoplastic transformation and progression and can cause also immune-evasion (103). Differentially to TAAs, tumor neoantigens (TNAs) originate from non-synonymous point mutations, rearrangements as well as frameshift mutations caused by small insertions and deletions (indels) in proteins that are expressed and properly processed by the major histocompatibility complex (MHC) molecules, the antigen presentation machinery (103).These TNAs differ from self-epitopes and, once exposed on the surface of malignant cells, can efficiently prime de novo immune responses (104). TNAs are more powerful in eliciting anticancer immunity than TAAs, but not all tumors have a mutational burden sufficient to lead to TNAs expression (104). It has been shown that, although TAAs are weaker than TNAs in immune anticancer stimulation, they can be relevant for ICD-driven immunity in tumors with low TNA load (104). If on one hand the high mutational burden of certain tumors is positively correlated with antigenicity, on the other hand it can be also involved in the impairment of antigen presentations, thus avoiding immune system recognition. Indeed, during cancer evolution, tumor cells can lose antigen subjected to active immunity (104) and/or can show an alteration of key components of the antigen-presenting machinery caused by mutations, deregulated expression, or structural alterations (104).

In the context of ICD, tumor-related adjuvanticity is induced by the exposure, active secretion, or release in the extracellular space of numerous DAMPs and inflammatory cytokines. DCs, thanks to their expression of chemotactic receptors (102), can be recruited in tumor bed, where they can recognize cancer cells and properly maturate, thus triggering the activation of cytotoxic T lymphocyte (CTL)-driven adaptive immunity. The crucial reported DAMPs involved in ICD induction are the ER chaperone calreticulin (CRT), ATP, the non-histone nuclear DNA-binding protein high mobility group box 1 HMGB1, the member of the annexin superfamily annexin A1 (ANXA1), cytokines like IFNs, C-X-C motif chemokine ligand 10 (CXCL10) (105) (**Figure 5**).

During ICD, the ER stress response is initiated, inducing the activation of eukaryotic translation initiation factor 2 alpha kinase 3 (PERK) that, in turn, phosphorylates the initiation translation factor eIF2α at serin 51, causing the blockade of protein translation; as a consequence of the induction of ER stress, the ER chaperone calreticulin (CRT) translocates from ER side to the outer leaflet of the plasma membrane, where it acts as an “eat me” signal DAMP for immune cells, by binding the CD91 receptor (LRP1) on the surface of DCs. (105). Of note, CRT exposure is necessary to made cell death immunogenic (105). Accordingly, Bezu *et al.*, reported that the phosphorylation of eIF2α at serin 51 (S51) is pathognomonic for ICD induction in the context of iatrogenic stressors (106); furthermore, a non- phosphorylable variant of eIF2α failed to evoke ICD (107).

As a consequence of cellular blebbing, ATP is released by cancer cells in the extracellular space (105), and it is recognized by two purinergic receptors on APCs, P2Y2 (P2RY2) and P2X7 (P2RX7). The former receptor favors the

recruitment of DCs and their precursors, while the latter favors DCs activation by stimulating the canonical inflammasome (105) (**Figure 5**).

Nucleic acids fragmentation produced during chemotherapy-driven ICD culminates in the in type I IFN secretion and the consequent initiation of an autocrine loop that generates CXCL10 (102). CXCL10 then acts as a chemoattractant for immune cells that express its cognate receptor CXCR3. Defects in this cascade abolish immunogenicity of cancer cell death in certain contexts (105).

The mechanism underlying ANXA1 and HMGB1 release during ICD remains to be fully understood. ANXA1, by interacting with formyl peptide receptor 1 (FPR1) on DCs, is able to spatially direct them to dying cancer cells (105). HMGB1, by binding advanced glycosylation end-product specific receptor (AGER) and Toll like Receptor 4 (TLR4), mediates potent proinflammatory effects (105). Upon HMGB1 binding, AGER receptor on DCs stimulates their maturation, while TLR4 activation promotes DCs antigen processing and cross-presentation (105).

In order to follow ICD induction by putative ICD inducers, Galluzzi *et al.* suggested to follow DAMPs induction upon specific single compound or combinatorial strategies treatment of cancer cells (108), and biochemical and functional assay to test the activation of DCs *in vitro*. Finally, they report that the gold standard feature for identification of *bona fide* ICD is the induction of antitumoral adaptive immunity by vaccination of immunocompetent mice with syngeneic tumor cells treated (*in vitro* or *in vivo*) with ICD inducers. The percentage of tumor-free mice and the growth rate of tumors are usually employed as indicators of (at least some) degree of immunogenicity (108).

Single compounds or combination therapies have been shown to function as ICD inducers in several tumor types. Some example of cellular stressors reported as ICD inducers are: conventional chemotherapeutics like anthracyclines and some DNA-damaging agents, (i.e., oxaliplatin, but not cisplatin), targeted anticancer agents, such as the tyrosine kinase inhibitor crizotinib, the epidermal growth factor receptor (EGFR)-specific monoclonal antibody cetuximab, numerous physical interventions, such as ionizing radiation, hypericin-based photodynamic therapy (PDT), high hydrostatic pressure and finally other chemicals including the chinese herbal medicine component shikonin and capsaicin (105,108,109).

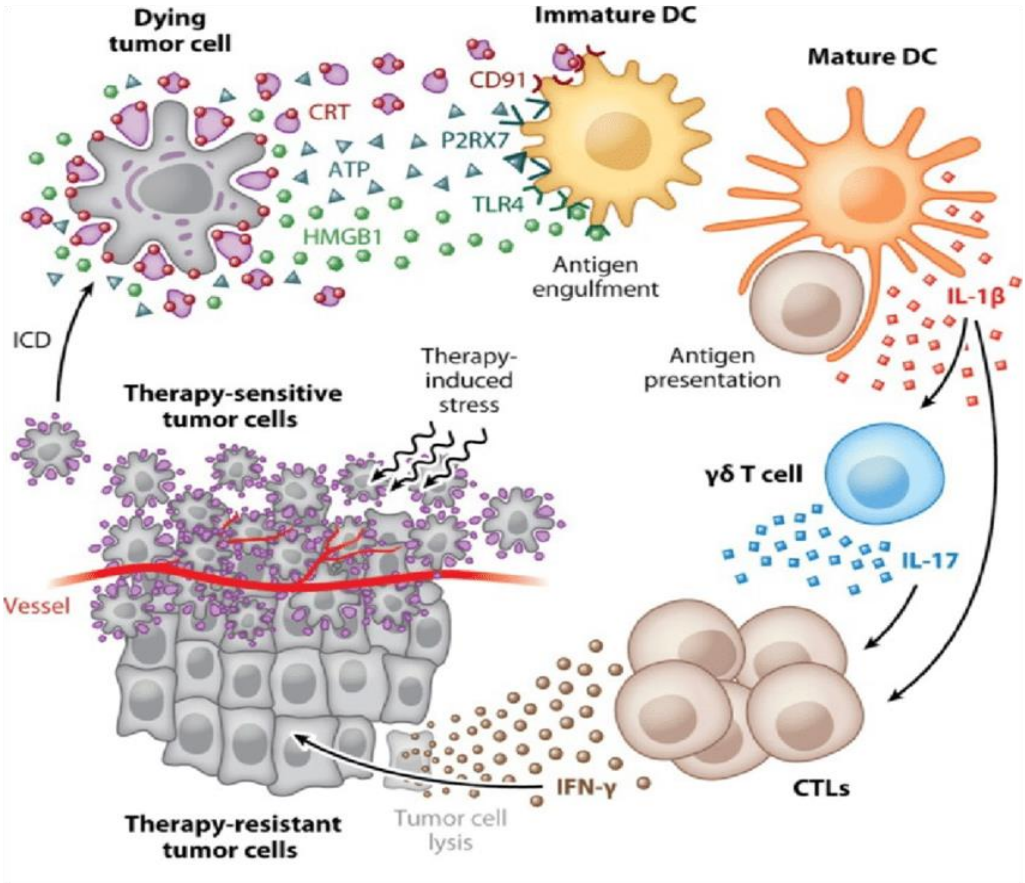

Figure 5. Properties of immunogenic cell death (ICD). As a result of premortem endoplasmic reticulum stress, cancer cells responding to ICD inducers expose CRT on the outer leaflet of their plasma membrane at a preapoptotic stage and secrete ATP during apoptosis. In addition, cells undergoing ICD release the nuclear protein HMGB1 as their membranes become permeabilized during secondary necrosis. CRT, ATP, and HMGB1 bind to CD91, P2RX7, and TLR4, respectively. This facilitates the recruitment of DCs into the tumor bed (stimulated by ATP), the engulfment of tumor antigens by DCs (stimulated by CRT), and optimal antigen presentation to T cells (stimulated by HMGB1). Altogether, these processes result in a potent IL-1β- and IL-17-dependent, IFN-γ-mediated immune response, involving both γδT cells and CTLs, which eventually can lead to the eradication of chemotherapy-resistant tumor cells. (Abbreviations: ATP, adenosine triphosphate; CRT, calreticulin; CTL, cytotoxic CD8+ T lymphocyte; DC, dendritic cell; HMGB1, high-mobility group box 1; IFN, interferon; IL, interleukin; TLR, Toll-like receptor.). From Kroemer *et al.*, 2013 (105).

2. AIMS

Anaplastic Thyroid Carcinoma (ATC) is the most aggressive and chemo-resistant phenotype of follicular cell derived TC, whose management still remains challenging.

Despite immune system is able to control cancer growth, it is well established that cancer cells can overcome immune system attack by diverse mechanisms (87). One of the best characterized is the expression, in the context of cancer, of immune checkpoints (ICs), including programmed death 1 (PD-1) and its ligands programmed cell death-ligand 1 and ligand 2 (PD-L1, PD-L2) (87). Cancer cells can bypass anticancer immune response by exploiting the PD-1 circuit (87).

We recently demonstrated that IL8, produced both by cancer cells and immune cells infiltrating tumor bed, is able to sustain TC aggressiveness by favoring cancer cell proliferation, survival, migration, induction of EMT and stemness feature (63,85). Interestingly, preliminary results demonstrated that IL8 is also able to potentiate TC cell immunosuppression as demonstrated by the reduction of T lymphocyte proliferation and survival in co-culture experiments. We found that this immune suppression was mediated by the ability of IL8 to increase the expression of the PD-1 ligands PD-L1/2 on TC cells. Interestingly, we found that PD-1 is also constitutively expressed by TC cells in culture and in TC surgical samples.

Starting from the above-mentioned observations, here we explored the role of the immune checkpoints PD-1, PD-L1 and PD-L2 in TC context with a particular attention to the PD-1 intrinsic signaling. We investigated PD-1 activation effects on TC cells and the downstream mechanisms of action.

Based upon previous data, and on data presented in this thesis, both IL8 and PD-1/PD-L1 represent potential therapeutic targets in ATC context. Thus, we exploited the possibility to use compounds targeting IL8 or PD-1, alone or in combination, to define novel strategies that could induce ICD in ATCs.

3. MATERIALS AND METHODS

3.1 Cell culture and transfection

The normal thyroid cells H6040 have been isolated from normal human thyroid tissue and cultured in Human Epithelial Cell Medium with the addition of Insulin-Transferrin-Selenium, EGF, Hydrocortisone, L-Glutamine, antibiotic-antimycotic solution, Epithelial Cell supplement, and FBS were obtained from Cell Biologics (Chicago, IL, USA). Human thyroid cancer cell lines, TPC-1, BcPAP, 8505c and SW1736 were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine (Life Technologies Inc., Pasley, PA)

TT2609-Co2 cells were maintained in RPMI 1640 supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine (Life Technologies Inc., Pasley, PA)

FTC133 cell line was maintained in DMEM/HAMF12 (1:1) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine (Life Technologies Inc., Pasley, PA)

Murine thyroid cancer cell line T683 (110) was kindly provided by A. Di Cristofano and maintained as elsewhere described (110).

All cell lines were kept in incubator at 37°C in humidified atmosphere containing 5% CO2.

PD-1 was cloned in pFLAG 5A (Invitrogen, Carlsbad, CA, USA). Transient transfections of 8505c cells were performed using polyethylenimine according to manufacturer's instructions (Merck, Darmstadt, Germany) (111).

3.2 Reagents

Soluble PD-L1 (sPD-L1) was from R&D systems (Minneapolis, MN, USA),
Nivolumab was kindly provided by S. Scala. IgG_4 control antibodies were from Invitrogen (Invitrogen, Carlsbad, CA, USA). SHP099 and Reparixin were from Selleckchem (Houston, TX, USA).

3.3 Cytofluorimetric analyses

Cells were incubated (30 min at 4 °C) with specific or isotype control antibodies, and then analyzed with a FACS Calibur cytofluorimeter using CellQuest software (BD Biosciences, Mississauga, ON, Canada). 10^4 events for each sample were acquired (111). Human anti-PD-1 and anti-PD-L1 were from ebioscience (Thermo Fisher, Waltham, MA, USA), anti-PD-L2 was from Miltenyi Biotec (Bergisch Gladbach, Germany) (111). Murine anti-PD-1, anti-CXCR1 and anti-CXCR2 were from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CRT Ab was from Novus Biologicals (Centennial, CO, USA) (112). The concentration used for flow cytometric staining was that indicated by manufacturers.

Detection of ANXA1 intracellular protein levels were performed by cell membrane permeabilization using the Cytofix/Cytoperm kit (BD Biosciences, Canada). ANXA1 staining was carry out using a primary anti-ANXA1 goat polyclonal Ab, at the concentration indicated by manufactures, (R&D Systems,

Minneapolis, MI, USA) followed by the staining with a secondary anti-goat Ab Alexa Fluor 488 (Invitrogen, Waltham, MA, USA). In this case the secondary antibody alone was used as a negative matched control (113).

3.4 S-phase entry

The incorporation of Bromodeoxyuridine (BrdU) in the DNA was used to evaluate S-phase cell entry. Cells were serum-deprived and treated with stimuli for 24 h. BrdU was added at a concentration of 10 μM for the last 1 h. BrdU-positive cells were revealed with Texas Red conjugated secondary Abs (Jackson Laboratories, West Grove, PA, USA) (111). Fluorescence was detected by FACS analysis (86).

3.5 Migration Assay

Chemotaxis was evaluated using a Boyden chamber. A 48-well microchemotaxis chamber (NeuroProbe, Gaithersburg, MD, USA) and 8-μm-pore polycarbonate membranes (Nucleopore, Pleasanton, CA, USA) coated with 10 μg/ml fibronectin (Merck) was used, as described elsewhere (111).

3.6 Protein studies

Protein extraction and immunoblotting experiments were performed according to standard procedures (111). Antibodies to PD-1, phospho-BRAF, phospho-MEK1/2, phospho-MAPK (p44/p42), SHP2, phospho-EIF2α and total EIF2α for Western blot analysis were obtained from Cell Signaling Technology (Danvers, MA, USA), anti-tubulin antibody was from Sigma Aldrich. Secondary anti-mouse and anti-rabbit antibodies were linked to horseradish peroxidase (Biorad, Hercules, CA, USA).

Total 8505c cells lysates were subjected to reciprocal coimmunoprecipitation with anti-PD-1 or anti-SHP2. The immunocomplexes were eluted and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with indicated antibodies (111).

Enhanced chemiluminescence (ECL; Thermo Fisher) was used for immune detection of target proteins or proteins complexes (111).

3.7 MTT Assay

8505c and T683 cells were plated in 96-well plates at a density of 1500 cells/well. The day after seeding the cells were treated or not with the above-mentioned treatments. After 48 hs cell viability was determined by using MTT assay (Promega). The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a salt that is reduced to formazan, a violet-blue water-insoluble molecule, only by metabolically active cells (114). Then formazan was solubilized by Dimethyl sulfoxide (DMSO and the absorbance (around 570 nm) was detected by optical microplate reader Biotek Synergy H1 (Agilent, Santa Clara, CA, USA) (114).

3.8 Clonogenic Assay

8505c and T683 cell lines were plated in duplicates at the density of 1.5×10^3 and 0.5×10^2 cells/well respectively, into six-well plates, incubated for 24 hs, and then treated or not with Nivolumab (1 μg/mL) alone SHP099 (3 μM) alone or Reparixin (10 μM for 8505c cells and 30 μM for T683 cells) or their above-mentioned combinations for 14 days. Then, cells were stained with crystal violet. Quantitative estimation of the number of colonies was performed by counting clustered pixels (1 pixel is referred to 1 cell) using ImageJ (115). A population of more than 50 cells was scored as one survivable colony (116).

3.9 ATP release

The cells were treated or not with Nivolumab (1 μg/mL) alone SHP099 (3 μM) alone or Reparixin (10 μM for 8505c cells and 30 μM for T683 cells) or their above-mentioned combinations and ATP release was tested in the supernatant after 24 hs by using ENLITEN ATP Assay System (Promega, Madison, WI, USA) following manufacturer's instructions. This assay uses recombinant luciferase to detect ATP based on the following reaction: ATP + D-Luciferin + O_2 → Oxyluciferin + AMP + PPi + CO_2 + Light (560 nm). Chemiluminescence was detected by optical microplate reader Biotek Synergy H1 (Agilent, Santa Clara, CA, USA) (117).

3.10 HMGB1 release

8505c and T683 cell lines were treated or not with Nivolumab (1 μg/mL) alone SHP099 (3 μM) alone or Reparixin (10 μM for 8505c cells and 30 μM for T683 cells) or their above-mentioned combinations and HMGB1 release was tested in the supernatant after 24 hs by HMGB1 ELISA, according to manufacturer's instructions (IBL International, Hamburg, Germany) (109). Absorbance at 450 nm was detected by optical microplate reader Biotek Synergy H1 (Agilent, Santa Clara, CA, USA).

3.11 Statistical analysis

The results are expressed as the mean ± SD of at least 3 experiments. Values from groups were compared using the paired Student *t* test or Duncan test.
P value < 0.05 was considered statistically significant.

4. RESULTS

5.DISCUSSION
6. CONCLUSIONS

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8. LIST OF PUBLICATIONS

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