Universidade de Aveiro Departamento de Química
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Contribuição do stress do RE para o microambiente imunossupressor do tumor

Contribution of ER stress to tumor immunosuppressive microenvironment

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, especialização em Bioquímica Clínica, realizada sob a orientação científica do Doutor Bruno Miguel Rodrigues das Neves, Professor Auxiliar Convidado do Departamento de Química da Universidade de Aveiro, e da Doutora Luisa Alejandra Helguero, Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro.

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o júri

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Palavras-chave

Stress do Retículo Endoplasmático Transmissível, células tumorais, mecanismos de imunoevasão, células dendríticas, exossomas

Resumo

O cancro da mama é o cancro de maior incidência entre as mulheres, sendo também uma das situações oncológicas que mais mortes causa. Na última década inúmeros estudos têm demonstrado que os tumores sólidos geram um microambiente favorável à evasão/subversão do sistema imune. Esse microambiente (acidose, hipoxia, deprivação de glucose, citoquinas) é muita das vezes propicio à indução de *stress* do reticulo endoplasmático (RE). O *stress* do RE é primariamente uma resposta no sentido de restabelecer a homeostasia no entanto se não resolvido resulta normalmente na morte celular por apoptose. O *stress* do RE e a respetiva resposta às proteínas mal conformadas (UPR), desempenham um papel paradoxal na fisiopatologia do cancro: os três ramos da UPR, PERK, IRE1 e ATF6, contribuem ativamente para a sinalização de alguns mecanismos de sobrevivência e metastização. Recentemente, foi descrita uma possível transmissão do *stress* do RE das células tumorais para as células do sistema imunitário, modulando a ação destas. Desta forma, pretendeu avaliar-se com o presente trabalho a capacidade e os mecanismos pelos quais células tumorais T-47D transmitem o *stress* do RE para células monocíticas THP-1, e quais as consequências desta transmissão. A transmissão foi apenas observada aquando da utilização de indutores farmacológicos como a tunicamicina, não se registando para estímulos fisiológicos como a deprivação de glucose. Por outro lado, verificou-se que a tunicamicina parece ser transportada via exossomas e desta forma induzir diretamente *stress* do RE nos monócitos. Observou-se ainda que os exossomas provenientes das células T-47D em *stress* do RE por deprivação de glucose apesar de não transmitirem o referido *stress* conduzem os monócitos para um perfil pró-inflamatório específico diminuindo ainda a sua capacidade de maturação. Em geral, os nossos resultados questionam seriamente o mecanismo de transmissão de *stress* ER tal como originalmente descrito, mostrando que no uso de indutores farmacológicos o que parece ocorrer é o transporte do fármaco em vesículas e a indução direta nas células recetoras.

Keywords Transmissible endoplasmic reticulum stress, unfolded protein response, tumor cells, immunoescape mechanisms, dendritic cells, exosomes

Abstract

Breast cancer is the most prevalent cancer among women and also one of the oncologic pathologies that causes more deaths. In the last decades several studies have reported that solid tumors generate an immunosuppressive microenvironment. This microenvironment (acidosis, hypoxia, glucose deprivation and cytokines) is favourable to endoplasmic reticulum (ER) stress induction. ER stress is primarily a response towards the re-establishment of homeostasis; however if not resolved it usually results in cell death by apoptosis. Nevertheless, ER stress and unfolded protein response (UPR) play a paradoxical role in cancer physiopathology: the three branches of UPR, PERK, IRE1 and ATF6 actively contribute to signalling of survival and metastasis mechanisms. Recently it was reported a possible transmission of ER stress from tumor cells to immune cells, modulating the phenotype and function of recipient cells. Thus, the aim of the present work is to assess the ability and the respective mechanisms by which T-47D tumor cells transmit ER stress to THP-1 monocytes, and the consequences of this transmission. ER stress transmission was only observed when pharmacological ER stress inducers were used, such as tunicamycin, contrarily to physiological stimulation, as glucose deprivation. Additionally, it was found that tunicamycin seems to be transported within exosomes which, in turn, directly induces ER stress on monocytes. It was also observed that exosomes derived from glucose deprived T-47D cells do not transmit ER stress; however these exosomes conduct monocytes towards a particular proinflammatory profile, accompanied by the decrease of its maturation status. Overall, our results question the ER stress mechanism originally described, showing that pharmacological ER stress inducers can be transported within exosomes and directly inducing ER stress on recipient cells.

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Abbreviations

APC	Antigen-presenting cell
ASK1	Apoptosis signal-regulating kinase 1
ATF6	Activating transcription factor 6
B7-H1	B7 homolog 1
BAGE	B melanoma antigen
BC	Breast cancer
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophages
BRCA1/2	Breast cancer 1/2
CD81/63/86	Cluster of differentiation 81/63/86
cDC	Conventional dendritic cell
C/EBP	CCAAT-enhancer-binding proteins
CHOP	C/EBP homology protein
CLP	Common lymphoid precursors
CM	Conditioned medium
CMP	Common myeloid precursors
CNX	Calnexin
CRT	Calreticulin
CTL	Cytotoxyc T Cell
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CXCL12/14	Chemokine (C-X-C motif) ligand 12/14
DC	Dendritic cell
DCIS	Ductal carcinoma *in situ*
EDEM	ER degradation enhancer mannosidase
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERP	Exported Repetitive Protein

ERSE	Endoplasmic reticulum stress elements
EXO	Exosomes
FOXP3	Forkhead box P3
GADD153	Growth arrest and DNA damage-inducible protein
GADD34	DNA damage 34
GCN2	General Control Nonderepressible 2
GM-CSF	Granulocyte macrophages colony-stimulating factor
GRP	Glucose-related protein
HER2	Human epidermal growth factor receptor
HIF-1	Hypoxia inducible factor 1
HMOX	Heme Oxygenase
HSC	Haematopoietic stem cell
HSP	Heat-shock protein
HRI	Heme-Regulated eIF2α Inhibitor kinase
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon-α
IkB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IKK	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor kinase
IL-	Interleukin-
IRE1	Inositol-requiring kinase 1
JNK	C-Jun N-terminal *kinase*
Lact	Lactate
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein 1
M-CSF	Macrophage Colony-Stimulating Factor
MDSC	Myeloid-Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MMP	Matrix MetalloProteinase
Mo	Macrophages
mRNA	messenger RNA

MSDC	Myeloid Suppressor-derived Dendritic Cell
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NK	Natural killer cell
NKT	Natural killer T cell
OER	Oestrogen receptor
PD	Pyruvate dehydrogenase
pDC	Plasmocytoid dendritic cell
PDI	Proteins dissulfide isomerase
PERK	Protein kinase-like ER kinase
Phe	Phenantroline
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKR	Protein Kinase R
PR	Progesterone receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S1(2)P	Site 1 (or 2) protease
TAM	Tumor-associated macrophages
TCR	T cell receptor
TEM	Transmission Electron Microscopy
TERS	Transmissible endoplasmic reticulum stress
TGF-β/α	Transformation growth factor-β/α
Th	T helper cell
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6
TNF-α	Tumor necrosis factor-α
TRAF2	TNF receptor-associated factor 2
TRB3	Tribbles homolog 3
Treg	T regulatory cell
uORF	upstream Open Reading Frame
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau tumor suppressor
XBP1	X-box binding protein 1

Introduction

1. Cancer

Cancer is one of the leading causes of death, accounting for about 13% of all deaths in the world in 2008 [1]. Actually, about 70% of worldwide cancer-related deaths are in low and middle-income countries given that, in developed countries, the easiest access to innovative therapies greatly contributes to lower the mortality [1]. Cancer is the common designation for a family of diseases characterized by the uncontrolled growth of cells from some tissue, resulting in a loss of differentiation and altered control of cell cycle. However, this condition is only called as cancer when the growing tumor has a malignant character. Malignancy is characterized by loss of cell aggregative capacities, where cells detach from the extracellular matrix and spread to other body organs. These malignant cells, when they metastasize, will influence the correct growth, differentiation and metabolism of other cells and tissues, and, because of that, the tumor will be held not only at the origin site, but all over the body, including vital organs [2].

The leading cause of this abnormal cell transformation was traditionally studied as a genetic issue. Mutations are common in some proliferative-regulatory genes, such as proto-oncogenes (growth factors, receptors, signal transducers, transcription factors, cyclins) responsible for the progression of the cell cycle, tumor suppressors (transforming growth factor - β (TGF-β), E-caderin, β-catenin, p53, BRCA1 and BRCA2), and apoptosis-regulatory genes [3]. Therefore, during many years researchers focused their work on the fact that the loss of control was only result from genetic somatic mutations, and experiments were mainly performed using only purified malignant cells (either primary or established cell lines). However, over the last 15 years the studies started to focus on the influence of surrounding stromal cells on the breast carcinoma behaviour (reviewed in [4]). This opened a new vision to the cancer research: the tumor microenvironment contribution. Thus, the cellular proliferation is not an intrinsic process by itself, as surrounding cells also contribute to the tumor modulation, by releasing cytokines, chemokines, inflammatory mediators and extracellular enzymes, such as matrix metalloproteinases (reviewed in [4]). The tumor mass is composed not only by these abnormal proliferating cells but by a pool of other cell types as immune cells (macrophages, mast cell, neutrophils, natural killer cells (NK cells), myeloid-derived suppressor cells (MDSC), dendritic cells (DC), B and T

lymphocytes) and stromal cells as fibroblasts, mesenchymal and endothelial cells. The tumor microenvironment is also very rich in soluble factors such as growth factors, cytokines and chemokines, molecules of upmost importance in the intercellular communication, particularly to tumor-stromal cells [5]. The expression and secretion of these factors is a potent way by which tumor cells can control their growth or death, depending on the balance between pro- and anti-inflammatory signals. Moreover, tumor microenvironment is also characterized by its particular and extreme conditions. The abnormal fast proliferation of cancer cells, results in an insufficient perfusion of oxygen, glucose or amino acids and inefficient clearance of the toxic cell products such as lactate, and carbon dioxide (CO_2). Cancer cells, characterized by the high capacity to adapt to the most noxae conditions, redirect their metabolic processes in order to obtain energy in low pO_2: the ATP production is mainly via glycolytic-lactate pathway [6]. Additionally to this environmental adaptation, researchers have also reported a specific feature of metabolism on these tumor cells: the Warburg effect. Warburg effect is observed in tumor cells that, even in normoxic conditions, produce ATP by glycolysis-lactate pathway. This process is explained by the dysfunction observed in cancer cells mitochondria, resulting from the action of high level of ROS in the tumor [7–9]. Thus, even if less efficient, the tumor cell is able to continue producing energy and substrates for nucleic acid, protein and fatty acid synthesis in hypoxia conditions and in a quicker way, enabling the overwhelming proliferation process. In this process several transcription factors, such as hypoxia-inducible factors (HIF-1) pay a central role. HIF-1, is activated either by hypoxia or by oncongenic pathways already activated like PI3K or mutations in tumor suppressor proteins such as von Hippel-Lindau (VHL) [10]. When activated, HIF-1 promotes the transcription of pyruvate dehydrogenase kinase (PDK) which in turn, will inhibit PD, preventing the formation of acetyl-CoA in the mitochondria and redirecting the glycolytic pathway through the lactate final product [11]. Moreover, activated HIF-1 also induces the transcription of GLUT-family transporters, which facilitate glucose import to the cell, providing more energy substrate [7].

1.1. **Breast Cancer**

Breast Cancer (BC) is the most frequent cancer in women (22,9% of all cancers) but if diagnosed and treated on time, a woman has a 90% chance to be cured [1]. This type of

cancer is characterized by a solid malignant tumor, and can be classified as invasive and non-invasive (*in situ*). *In situ* mammary carcinoma is designed when the tumor is in its initial stage, when there is no evidence of invasion of these tumor cells on other tissues (metastasis) or disorganization of myoepithelial cell layers and basement membrane [12]. Apart from that, breast cancer can be distinguished also by its origin: ductal (when it is developed from the inner lining of milk ducts) or lobular (when the tumor originating cells are from lobules that supply ducts with milk) [2]. The most common breast cancer is the ductal carcinoma *in situ* (DCIS), which isn't life-threatening, but may increase the chance to suffer from invasive cancer in some years from the initial diagnosed form [13]. The characterization and selection of a breast cancer cell line model has a great importance and relevance to the study and comprehension of physiopathological mechanisms and development of more efficient therapeutics on breast cancer. The classification of these cell lines (Table 1) is based on their phenotypic profile, mainly their estrogen and progesterone receptor expression, but also human epidermal growth factor receptor 2 (HER2) [14]. The BC cell lines commonly used are MCF-7 and T-47D, due to their hormone responsiveness (positive to both estrogen and progesterone receptors).

Table 1 - Breast cancer cell lines classification by its respective phenotypic profile (estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Adapted from [14].

Classification	Immunoprofile	Ligand Responsiveness	Cell lines example
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	Endocrine responsive	MCF-7, T-47D, SUM185
Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	Endocrine and Trastuzumab responsive	BT474, ZR-75
Basal	ER^-, $PR^{-/-}$, $HER2^-$	Endocrine nonresponsive, most aggressive cancer type (high proliferative rate and metastatic behaviour)	MDA-MB-468, SUM190
HER2 enriched	ER^-, $PR^{-/-}$, $HER2^+$	Endocrine nonresponsive but Trastuzumab responsive	SKBR3, MDA-MB-453

These cell lines are therefore recognized by some specific tumor markers, and by their secretion and phenotypic profile. For instance, steroid receptors, as progesterone (PR) and estrogen receptors (ER), are among the most common tumor biomarkers. These two endocrine receptors can be overexpressed in some particular BC cell lines, as luminal A and luminal B, and when bound to its ligands (17β-estradiol or progesterone, respectively) they promote cell growth and proliferation [15]. This fact opened some hope to endocrine therapies with the aim to inhibit these two receptors and researchers have developed estrogen receptor antagonists such as tamoxifen. In some more specific cases, as in $HER2^{+}$ cells, HER2 is a specific receptor with tumor biomarker properties. It is expressed in 25-30% of breast cancer cases, and one inhibitor was developed and shown to stop tumor growth and progression, trastuzumab. Trastuzumab is a monoclonal antibody, developed to bind with high affinity to HER2, preventing the activation of different pathways associated with the cell proliferation and anti-apoptotic mechanisms [16]. Another two important proteins that must be highlighted are E-cadherin and cathepsin-D, related to the stromal-epithelium interactions. Cathepsin is an oestrogen-induced lysosomal protease which has mitogenic activity on ER-positive breast cancer cell lines and a proteolytic activity on basement membranes and proteoglycans. This protein plays an important role on metastatic activity, being a biomarker of a poor prognosis [17]. Additionally, E-cadherin is a transmembrane protein, important to the cell adhesion and cellular migration. Loss of E-cadherin can cause dedifferentiation and metastatic invasiveness of tumor cells, and its presence is viewed as a good biomarker for certain types of cancer (lobular carcinomas) [18].

Other well-known biomarkers for the recognition of breast cancer cell's behaviour are involved in cell cycle deregulation (cyclin D1, p16NK4a and p14ARF) and other metastatic factors (vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), epidermal growth factor receptor (EGFR) and TGF-α [4].

1.1.1. Breast cancer microenvironment

Mammary carcinoma, apart from its origins (ductal, lobular or other) is a solid tumor with distinct particular phenotypic characteristics. For example, Polyak and collaborators have compared the genes corresponding to secreted proteins in ductal carcinoma *in situ* with normal epithelium and concluded that transcription profiles are substantially different.

Some genes were found to be upregulated, such as *HLA-C I* and II, *CXCL14* and *CXCL12*, matrix metalloproteinase-2 (*MMP2)* and *TNFAIP6* [6]. MMPs are crucial to the ECM remodelling and the upregulation of MMP2 reflects the malignancy of these cells towards the invasive and metastatic properties. In the same line of thought, the chemokine CXCL12 is highly associated with growth, invasion and metastasis of cancer cells (reviewed in [6]). CXCL14 is also a chemokine and has the capacity to attract antigen-presenting cells such as dendritic cells and NK cells. By this mean, we can evidence the different targets of tumor-induced factors: CXCL12 is a pro-tumorigenic and CXCL14 an anti-tumorigenic factor. *TNFAIP6* is a gene which encodes for a secreted protein involved in extracellular matrix stability and migration. It is induced mainly by tumor necrosis factor-α (TNF-α) and interleukine-1β (IL-1β) [19]. Cytokines are small glycoproteins greatly involved in inflammatory and immunological processes, and on tumor microenvironment they markedly influence the cancer cells behaviour [20]. Some of the most studied cancer-involved cytokines are IL-1β, TNF-α and IL-6, which are present in high levels in the tumor microenvironment. These cytokines have potent proliferative properties as evidenced by experiments in ERα-positive breast cancer cell lines treated with IL-6 [21].

In spite of their primary protective function, tumor-infiltrating cells such as macrophages, monocytes and T lymphocytes (T cells) paradoxically express and secret pro-tumorigenic factors. This fact gave a new focus to the researchers, the study of "tumor-induced cells", such as tumor associated macrophages (TAM) (Figure 1). TAM result from the differentiation of monocytes within the tumor microenvironment. In fact, evidences showed that the aggressiveness of carcinoma and poor prognosis is directly associated with the higher levels of TAM [22]. TAM´s most expressed cytokine is TNF-α, and even if it seems highly cytotoxic to the tumor, it was observed that chronic levels of TNF-α in the microenvironment actually promotes tumor growth (by inducing expression of MMPs, adhesion molecules and reactive oxygen species) [6]. Also T regulatory cells (Treg) have influence on tumor growth, as they are able to secrete anti-inflammatory interleukins as IL-4, IL-10, IL-13 and TGF-β. These cytokines suppress the anti-tumorigenic immunity mediated by natural killer (NK) cells and cytotoxic $CD8^+$ T cells and will also modulate DC maturation process [23].

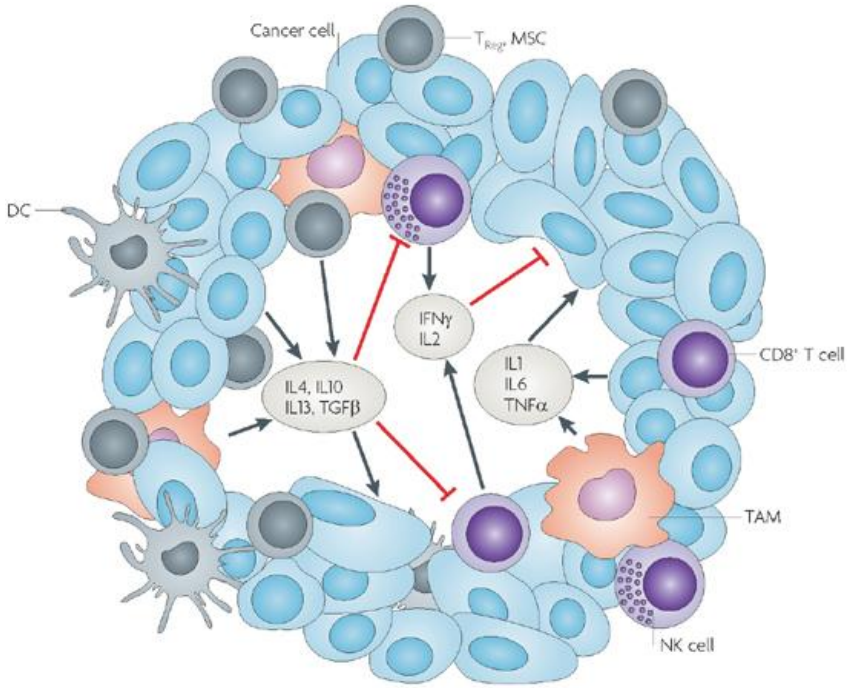


Figure 1 - Cancer cells and respective microenvironment, composed by tumor-associated macrophages (TAM), T-regulatory cells (Treg), dendritic cells (DC) and myeloid suppressor cells (MSC). The secreted cytokines are represented also as well as the respective inhibited/activated targets. Taken from [23].

2. Immune system and cancer

As referred above, tumor cells and their microenvironment have particular characteristics that perturb the homeostasis of tissues influencing the normal function of other cells. To prevent the loss of homeostasis caused by pathogens, inflammatory cells and/or tumor cells, the immune system is activated and is responsible for the management of those threats.

The immunogenic properties of cancer cells have been discussed for decades and two main theories have been presented: the self-nonself theory which persisted for more than 60 years and the most recent one, the danger model. The self-nonself theory was suggested by Burnet in 1949 and postulates that immature antigen-presenting cells (APCs) act as sentinels which are sensitive to genetic foreign entities, such as pathogens (reviewed in [24]). However, this theory couldn't explain why immune system is also involved in cancer, inflammation, injured cells and also why immune cells didn't attack commensal

bacteria that are not a threat to our body. In 1994, Matzinger suggested a renewed model to explain it: instead of APC recognize foreign entities, they were able to process danger signals (chemokines, cytokines, products from cell degradation, interferon-α (IFNα), IL-1β, heat-shock proteins (HSPs), among others) from intrinsic cell processes or from infected cells [25]. Despite the differences about the origin of the stimulus, the two models agreed that the stimulus is processed by non-activated/immature APCs which in turn present and prime naïve T lymphocytes triggering the expansion of effector cells, in a microenvironment dependent way.

2.1 Cancer immunosurveillance

APCs, such as dendritic cells and macrophages, are distributed to all tissues in their immature state, scanning their microenvironment for any change. When APC experience the presence of a tumor antigen, these cells are capable to endocyte the antigen, to process it and present it to adaptive immune cells. The processing step is crucial for the correct presentation and activation of T cells and it can proceed in 4 different pathways, depending on the origin of the antigen: a) endogenous peptides via MHC class I, b) exogenous antigens via MHC class II, c) exogenous antigens via MHC class I (cross-presentation) and d) lipidic antigens (endogenous/exogenous) via CD1 molecules [26]. In MHC class I presentation, endogenous proteins are targeted to proteosomal degradation and products proceed to endoplasmic reticulum to bind new synthetized MHC I molecules. This new complex (MHCI-antigen) migrates to the cell membrane, where lately they could interact with TCR receptor on $CD8^+$ T cells. On other hand, the exogenous antigens are endocyted and directed to phagolysosomes where antigen peptides are coupled with MHC II molecules. These complexes then migrate to the membrane by exocytic vesicles and are presented to $CD4^+$ T cells. Cross presentation, a unique feature of DCs *in vivo*, is a process where exogenous antigens are presented through MHCI molecules. This process is extremely important in anti-tumor immunity given that it leads to $CD8^+$ T cell activation into cytotoxic effectors that will destroy tumor cells. Finally, CD1-coupled lipid antigen pathway is based on APC-synthesized CD1 molecules which migrate to cytoplasmic membrane and bind to the lipid antigen, which can be either exogenous or endogenous. CD1-lipidic antigen complex is presented to natural killer T cells (NKT), also potent anti-tumor effective cells. Once activated, NKT cells produce large amounts of IFN-γ,

cytokines such as IL-4 and IL-13 and also the CD40 ligand. CD40 ligand binds to CD40 receptor in DCs and induces the expression of IL-12, CD80 and CD86, three essential molecules in the DCs-mediated antigen presentation to cytotoxic T cells (CTLs) (reviewed in [26]).

The interaction between TCR and CD1/MHC-antigen complexes on the APCs is the first and most important signal to initiate T cell priming. However, for effective T cell activation, another important second signal is required: the interaction of co-stimulatory molecules and its ligands such as CD28/B7 co-stimulatory system (Figure 2). CD28 is a co-stimulatory molecule present in the T cell membrane, and upon binding to its ligands CD80 and/or CD86, triggers T cells proliferation and differentiation, mainly by inducing IL-2 production [27]. The importance of this co-stimulatory signal was demonstrated in experiments where $CD28^{-/-}$ T cells were shown to have decreased sensitivity to antigen stimulation and a lower IL-2 production [28]. Therefore, the absence of CD28/B7 interaction during APC antigen presentation rather than induce T cell activation may induce anergy (antigen nonresponsivness). This co-stimulatory system is also involved in the tumor-induced immunosuppression, as we will describe it later.

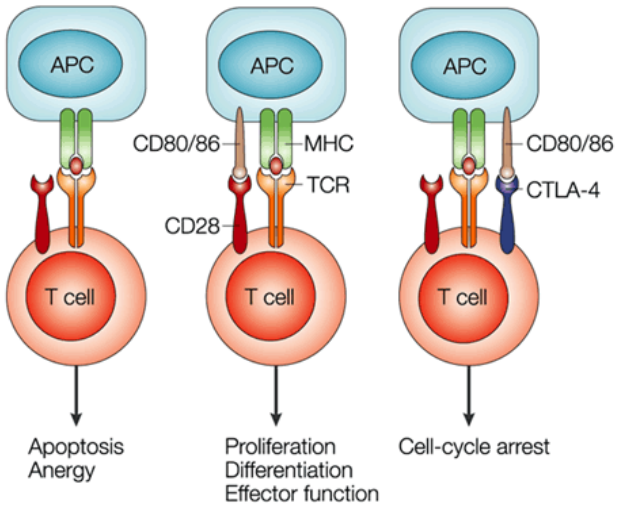


Figure 2 - T cell fates according to the different CD80/86 engagements. TCR/MHC-antigen is the first signal and is essential to the T cell activation. Even so, the engagement of the co-stimulatory protein CD80/86 with the respective receptor, CD28, is necessary. This results in the activation, differentiation and proliferation of cytotoxic T cells. However, if T cell possesses the inhibitory receptor CTLA-4 in its membrane, CD80/86 binds preferentially to CTLA-4, precluding the stimulatory engagement and leading to the cycle arrest in T cell. Taken from [29]

2.1.1 Dendritic cells in anti-tumor immunity

Among APC, dendritic cells are of most importance in the development of anti-tumor immunity. DCs are ubiquitously distributed among the organism and play crucial roles in driving adaptive immune responses and maintaining immune tolerance to self-antigens. They function as sentinels, scanning changes in their local microenvironment and transferring the information to the cells of the adaptive immune system. The importance of DCs on adaptive immune response was first evidenced by Steinman and his team in a series of pioneer experiments showing that these cells have unique abilities to prime adaptive immune responses. These discoveries lead to a new era of knowledge related to the integration between innate and adaptive immunity. Actually, DCs were viewed as a heterogeneous family of highly specialized antigen presenting cells that ultimately share the same hematopoietic stem cells (HSC) precursor. In bone marrow, HSC give rise to common myeloid precursors (CMPs) and common lymphoid precursors (CLPs), which are progressively engaged with a particular cell lineage (5). The enormous heterogeneity of DC population makes their classification rather complex, and these cells are often subdivided based on parameters such as differentiation stage, location in the body, migratory properties, expression of surface markers and activation stage. In humans, the classification is mainly based on surface markers expression, being DCs classified into two major subsets: conventional DCs (cDCs) and plasmocytoid DCs (pDCs). Conventional DCs are of myeloid origin being found in tissues and peripheral blood. They can be subdivided into $CD4^{+}CD1a^{+}CD11c^{hi}CD1c^{+}$ DCs, the direct precursors of *Langerhans* cells in the epidermis (17) and into $CD4^{+}CD1a^{-}CD11c^{low}CD141^{+}$ DCs, considered the precursors of dermal and mucosal DCs. These conventional DCs cells are predominantly present in peripheral tissues and play a major role in communicating the *in situ* danger to other cells of the adaptive immune system. (reviewed in [26]) In turn, pDCs have an enormous functional plasticity, being able to polarize Th1, Th2 and Treg. However, their main role is associated with anti-viral response, by producing high amounts of interferon type 1 (IFN-α/β) and by efficiently perform cross presentation of viral antigens to $CD8^{+}$ T lymphocytes (reviewed in [26]).

In a classical point of view, conventional immature DCs upon a "danger signal" suffer a complex process of morphological, phenotypically and functional modifications

referred to as maturation. These modifications allows their migration to the T-cell zones in the draining lymph nodes where, as activated DC, they present antigens to T lymphocytes [30]. The maturation process is a well-coordinated succession of events characterized by changes in morphology, loss of endocytic/phagocytic receptors, shift of chemokine receptors, resulting in acquisition of migratory capacity, upregulation of the co-stimulatory molecules CD40, CD54, CD80, and CD86, alteration of secreted cytokines/chemokines (IFN-γ, IL-10, IL-4, IL-13) but also proinflammatory ones (TNF-α, IL-1, IL-6, IL-12, IL-23) [31]. DC maturation can be triggered by multiple stimuli, including microorganisms or microorganism components, proinflammatory cytokines such as IL-1, IL-6, TNF-α and signaling molecules such as CD40 ligand (reviewed in [32]).

In the context of antitumor immunity, the capture/processing of tumoral antigens by DCs and the microenvironment where these events occur will be of upmost importance for the effectiveness of the immune response triggered. Tumor antigens may result from the phagocytosis and processing of apoptotic cancer cells or may be molecules secreted by cancer cells to their environment. These tumor antigens could be tumor-specific antigens (mutated or translocated genes such as *ras*, *β-catenin* or *cdk4*, overexpressed proteins like *p53* and *Her2/neu*) or tumor-associated antigens (abnormal or overexpressed proteins or genes not exclusive from tumors, such as *MAGE*, *BAGE* or *GAGE*) [24].

2.1.2 Tumor Subversion of DCs immunostimulatory abilities

Dendritic cells play a central role in priming cytotoxic cells and Th1 lymphocytes; therefore one of the main tumors immune escape mechanisms involves the subversion of DC immunostimulatory abilities. Although a large spectrum of tumor-mediated immunoescape mechanisms have been described (Figure 4) [33], the modulation of the different DC subsets is one of the most relevant . Tumor cells are capable to alter the DCs differentiation, through the release of granulocyte macrophage colony-stimulating factor, GM-CSF (which will act on bone marrow directly), but also through the high levels of VEGF, IL-6, macrophage colony-stimulating factor (M-CSF) TGF-β and ROS [32]. Additionally, DCs maturation is also modulated by tumor microenvironment, being normally impaired. The DC maturation process is crucial to biological functions given that, depending on their activation/maturation *status*, DCs polarize *naïve* T cells into their distinct effector and regulator subsets. The reduced expression of co-stimulatory molecules

such as CD80 and CD86 and the down-modulation of either MHC I/II molecules results on reduced T cell activation and even in T cell anergy [34]. Moreover, the high level of CTLA-4 in tumor microenvironment prevents antigen presentation by blocking CD86-CD28 interaction [35].

Another direct consequence of DCs maturation impairment by tumor cells is the development of T regulatory cells (Treg). Treg ($CD4^+CD25^+FOXP3$ or $CD4^+CD25^-$ FOXP3 T cells) are primarily responsible for promoting tolerance to non-dangerous self-antigens. This function is vital for the organism survival, as it prevents immune attacks towards healthy tissues. However, in the context of cancer, high levels of Treg were found in several tumor microenvironment being related to poor prognosis and tumor progression [36]. Several-tumor released cytokines and immunosuppressive molecules such as TGF-β, IL-10, IL-6 and VEGF directly contribute to the differentiation of DCs with tolerogenic polarizing profiles (reviewed in [26]). The presentation of tumor antigens by these tolerogenic DCs will lead to the priming and expansion of tumor antigen specific anergic T cells or Tregs. Tregs express CTLA-4, and FasL and secrete soluble factors such as IL-10, TGF-β, IL-35, granzyme B, and VEGF that will lyse or inhibit effector T cells (Figure 3), and supress immunostimulatory functions of NK and DCs [37]. To clarify the differentiation and phenotypic characteristics of tolerogenic DCs, Guerra and collaborators have stimulated monocyte-derived DCs with IL-10, IL-6 and TGF-β (simulating the tumor microenvironment) and observed that the resulted DCs had increased expression of IL-6 and IL-10, and lower secretion of proinflammatory cytokines such as IL-12 and IL-23 [38].

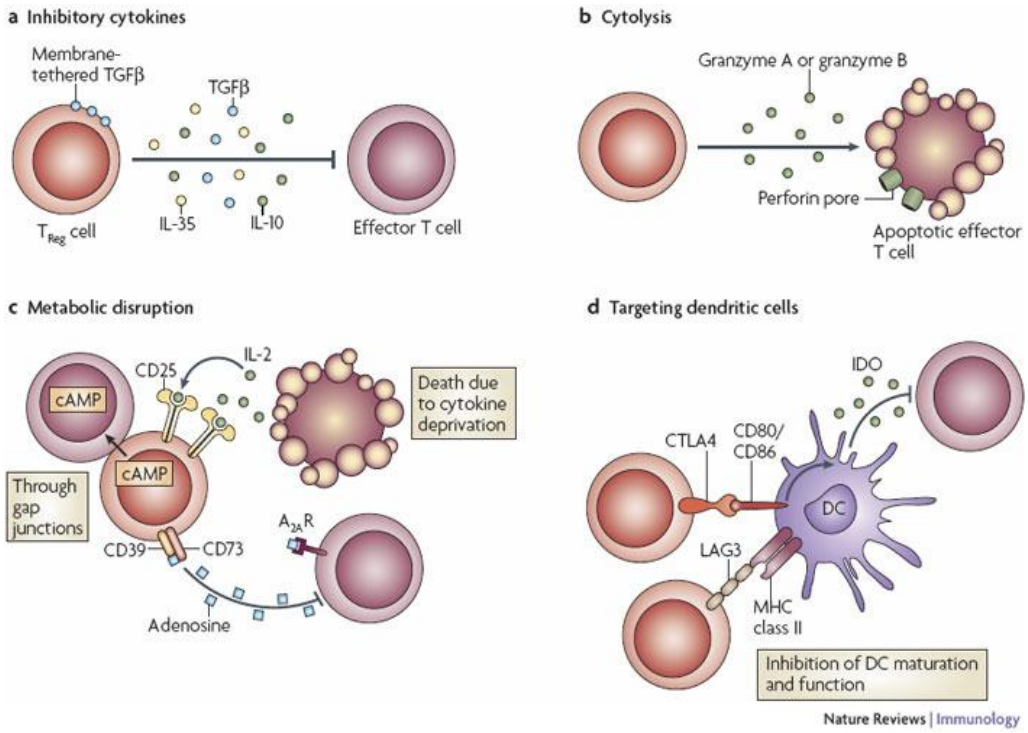


Figure 3 - Mechanisms by which Treg cells impair the function of DCs in the immunosuppressive tumor microenvironment. a) TGF-β and immunosuppressive ILs directly inhibit the cytotoxic action of activated T cells; b) granzyme A or B pass through perforine-mediated pores in T cell resulting in cytolisis of effector T cell; c) CD25 is a competitive receptor for the IL-12, necessary to the T cell activation. CD39 and CD73-generated adenosine inhibits T cells and direct transfer of cyclic AMP (cAMP) to T cells through gap junctions result in the suppression of T cells; d) T reg induced indoleamine 2,3-dioxygenase (IDO) that will act on effector T cells and induce the expression of pro-apoptotic molecules. Taken from [39]

Additionally, some studies showed that DCs from tumor environment had reduced ability to activate T cells not only because these cells expressed low levels of co-stimulatory molecules, but also because levels of viable DCs are lower than in healthy patients (reviewed in [40]). Supporting this fact, Lopez and co-workers found higher levels of apoptotic blood DCs on breast cancer patients compared to controls. They conclude that tumor-induced apoptosis resulted from downregulation of Bcl-2, an anti-apoptotic protein, adding another immunosuppresive mechanism to the subject [41].

Recent studies have described a new mechanism by which tumor cells may impair dendritic cell functions: cancer cells under endoplasmic reticulum stress release to their microenvironment several factors that transmit ER stress to neighbour cells including immune cells such as DCs. These ER stressed-DCs were shown to have deregulated maturation capacities and to possess limited stimulatory abilities [42,43]. This phenomena

was named transmissible ER stress (TERS) and is the main focus of the present work, being discussed in detail in the last chapter, *Paradoxical role of ER stress in cancer*.

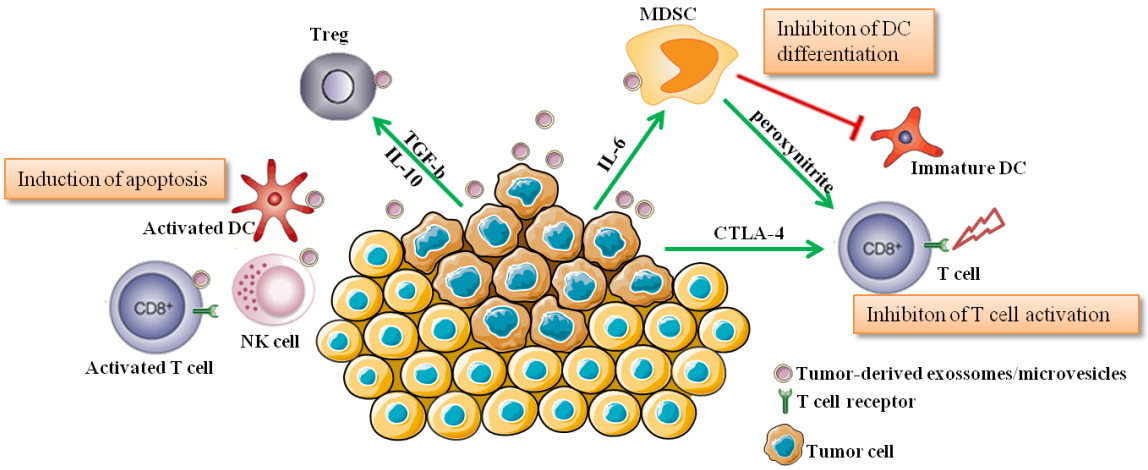


Figure 4 - Schematic view of the principal tumor-induced tolerance mechanisms. Despite of the anti-tumorigenic immune cells function, tumor cells are capable to modulate those mechanisms in benefit to the tumor survival and progression; the induction of apoptosis is the most well-known mechanism on cancer patients, which presented high levels of apoptotic immune cells on blood; the direct polarization of T cells in Treg, a suppressive phenotype; the inhibition of DC differentiation is mediated by the myeloid-derived suppressive cells, induced by the tumor-mediated IL-6; the direct influence on T cells, either by expressing CTLA-4, which blocks the co-stimulatory signal necessary to the T cell activation, but also by disrupting the TCR with the high levels of peroxinitrite. Some of these mechanisms are mediated by tumor-derived exosomes; however most of them are not clarified yet.

3. ER stress

Endoplasmic reticulum (ER) is a cellular constituent crucial to posttranslational protein modifications as it grants the proper protein folding. The effectiveness of protein-folding reactions depends on suitable environmental, genetic, and metabolic conditions and unbalances of these processes present a threat to cell viability and functionality. Conditions such as hypoxia, Ca^{2+} depletion, alteration on translational enzymes, glucose and/or amino acids deprivation, low pH, and changes on cell redox status can perturb ER homeostasis resulting in accumulation of unfolded proteins [44]. When ER stress occurs, healthy cells are able to detoxify misfolded proteins by activating an intrinsic response, known as unfolded protein response (UPR). UPR has two main steps: the first one evolves the maintenance of ER homeostasis, by slowing down translational process and also by chaperoning misfolded proteins (by GRP90, GRP78 or GRP94 upregulation) or even

ubiquitinated and degraded, by a process usually named by ER-associated protein degradation (ERAD) [45]. The ERAD process involves the expression of molecular chaperones that recognize and target misfolded proteins to cytoplasm where they are ubiquitinated and go through proteasomal degradation. Autophagy is also a mechanism that ER stressed cells make use to clear the non-resolved protein accumulation. The second step is the induction of apoptosis, when UPR recovery mechanisms aren't enough to re-establish the ER homeostasis and noxious stimuli are prolonged [45].

3.1 The Unfolded protein response

Proper adjustment to misfolded proteins requires modifications at multiple levels and cells developed complex mechanisms to avoid their accumulation. UPR is characterized by 3 parallel branches: inositol-requiring kinase 1 (IRE1), protein kinase-like ER kinase (PERK) and activating transcription factor (ATF6) pathways (Figure 5). The activation of UPR kinases will result on 1) slowdown the *de novo* entry of proteins in ER lumen 2) unfolded proteins repair to their functional form and/or 3) elimination of remaining abnormal proteins [46]. Each of these UPR-sensors is dependent of their binding/dissociation to the chaperone protein GRP78. When the protein synthesis rate is normal and efficient, the level of proteins on the ER lumen is low and the chaperone GRP78 is bound to IRE1, PERK and ATF6. However if ER homeostasis is unbalanced, efficient folding of proteins is compromised and misfolded proteins start to accumulate at ER lumen. In order to prevent formation of highly cytotoxic protein aggregates the chaperone GRP78 binds preferentially to those proteins, dissociating from the UPR-related kinases [46]. This dissociation triggers the phosphorylation and the activation of UPR pathways.

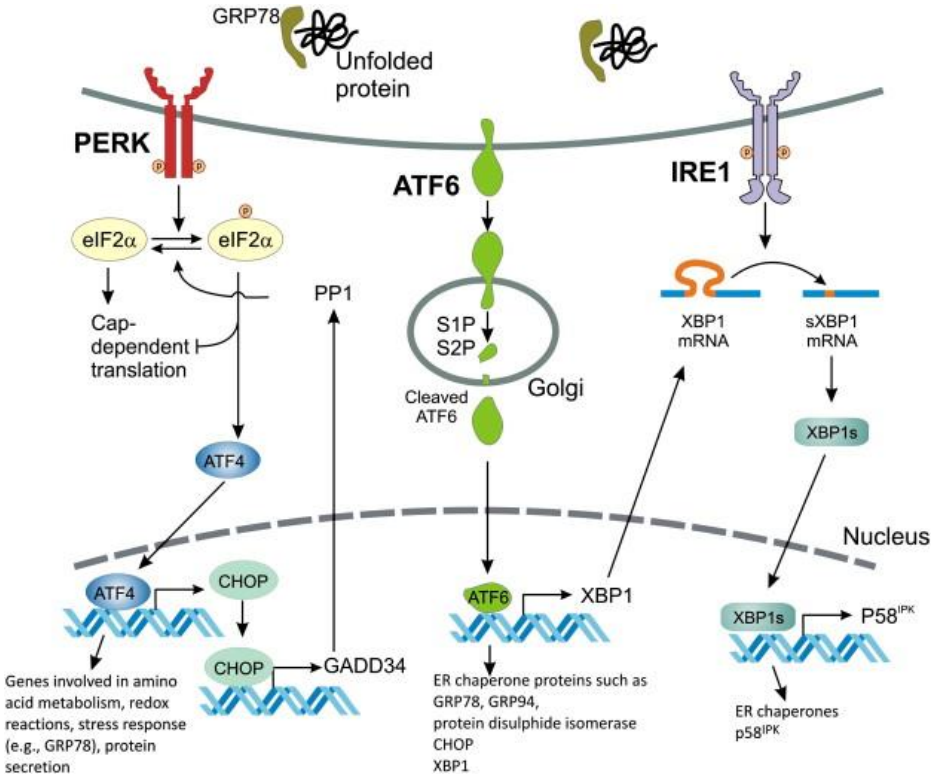


Figure 5 - Schematic view of the three branches of UPR in ER stressed cells. The activation of UPR-sensors is mediated by GRP78 association, and upon higher levels of misfolded proteins, GRP78 dissociates from PERK, ATF6 and IRE1. eIF2α phosphorylation by activated PERK selectively increases the translation of ATF4, an important transcription factor that upregulates specific genes such as chaperones and CHOP. ATF6 is cleaved in Golgi apparatus and the released cytosolic domain migrates to the nucleus where it activates the transcription of proteins involved in ERAD, refolding processes (as PDI) and also apoptosis (CHOP). IRE1 cleaves XBP1 mRNA and releases the activating transcription factors XBP1, also responsible for ER chaperones upregulation. Taken from [47].

3.1.1 IRE1α branch

IRE1α is a serine/threonine protein kinase with endoribonuclease activity. Its dissociation from GRP78 allows IRE1α to homodimerize and auto-transphosphorylate, resulting in activation of its endonuclease activity. From that stage, two signalling cascades can be activated 1) X-box binding protein 1 (XBP1) splicing that attenuates *de novo* protein entrance in stressed ER and 2) JNK-induced apoptosis. Spliced XBP1 translocates to the nucleus and initiate the transcription of ER-stress response elements (ESRE), essentially chaperones that promote the correct protein folding in ER. However, the most prominent transcriptional XBP1-targets are genes involved in ERAD process, chaperones molecules designed to conduct misfolded proteins to proteasomal degradation. The IRE1

can also interact and bind to tumor necrosis factor receptor-associated factor 2 (TRAF2) which activates apoptosis signal-regulating kinase 1, which in turn, activates JNK. In unstressed cells, TRAF is associated with inactive procaspase-12; however, under ER stress, TRAF2 dissociates from procaspase-12, releasing it into the cytosol. Procaspase-12 homodimerizes and suffer a cleavage that results in caspase-12 initiating an apoptotic cascade that culminates in cell death [48].

3.1.2 PERK branch

One of the main consequences of unfolded protein accumulation at ER is a rapid mRNA translation attenuation in order to prevent the influx of newly synthesized proteins. This translation arrest is mainly due to PERK-mediated phosphorylation of the eukaryotic translation initiation factor 2 at its α-subunit (eIF2α) [49].

PERK is a transmembrane protein kinase that following dissociation from GRP78, dimerizes and transphosphorylates, resulting on the active form. The kinase property will result on the phosphorylation of eIF2α. The phosphorylation of eIF2α subunit decreases the formation of translation complex, resulting in *de novo* protein translation attenuation. Although PERK-mediated phosphorylation of eIF2α inhibits the translational processes in general, it selectively activates the translation of one essential transcription factor of the UPR: the activating transcription factor 4 (ATF4). ATF4 induces the expression of stress related-genes, genes involved in amino acid metabolism, antioxidant response and apoptosis such as the DNA damage 34 (GADD34) and the growth arrest and DNA damage-inducible protein (GADD153)/CHOP [45]. In turn, CHOP suppresses the transcription of the anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-xL and Bcl-w. Other two CHOP targets are the activation of GADD34 which dephosphorylates eIF2α and inhibits PERK UPR-branch signalling and the activation of ER oxidoreductase (ERO1α) that develops an oxidizing environment in the ER, leading the cell towards death [50]. A growing body of data suggests that PERK branch of UPR, plays crucial roles in the protection of cells from oxidative stress (reviewed in [51]). Consistent with this notion, $PERK^{-/-}$ mouse embryonic fibroblasts showed to accumulate reactive oxygen species and to had a significant reduced survival to hypoxia stimulus when compared to wild-type fibroblasts [52].

3.1.3 ATF6 branch

Activating Transcription factor 6 (ATF6) is also involved on the UPR activation mechanisms. Upon ER stress, GRP78 releases from ATF6 to bind misfolded proteins on the ER lumen, allowing ATF6 to migrate towards Golgi apparatus for proteolytic cleavage by site-1 (S1P) and site-2 (S2P) proteases. S1P and S2P releases the cytosolic fragment of ATF6 which translocates into nucleus in order to activate the transcription of specific group genes: CHOP, ER chaperones and ERAD components [45]. The involvement of ATF6 branch in UPR-triggered apoptosis is not consensual; however, its role in ER stress recovery is well established, as it highly induces the expression of GRP78 and HSP90 chaperones [53].

3.2 Refolding, ERAD and autophagy mechanisms

In parallel to UPR, ERAD process is responsible for detoxifying stressed cells from misfolded proteins accumulated in ER lumen and, as UPR, re-establish the function of ER. This degradative process is characterized by 4 steps: 1) recognition, 2) retrotranslocation, 3) ubiquitination and 4) degradation. Despite the different goals of each step, they are all supported by chaperone proteins, namely GRP78, GRP94, protein disulphide isomerase (PDI), calnexin (CNX) and calreticulin (CRT) (reviewed in [54]). GRP78 and GRP94, as mentioned in the UPR system review, bind to the misfolded proteins with higher affinity compared to the UPR-sensors. In this way, GRP78 and GRP94 not only allow the UPR-sensors activation but also prevent the formation of misfolded proteins aggregates. GRP78 is one of the most studied proteins among ER stress and is well recognized as a ER stress biomarker (57–60). Moreover, among certain ER stress-related diseases, GRP78 levels can be used as diagnostic or prognostic marker. For example, in a study with 219 prostate cancer patients, it was observed an association between high levels of GRP78 and resistant tumor cells, as well as an enhanced risk of tumor recurrence and decreased patient survival [58]. This overexpression was also observed in mammary and virus-related hepatocellular carcinoma [56,59]. In rheumatoid arthritis salivary proteome, Giusti and collaborators found that GRP78 levels were increased about 7-fold in patients when compared to healthy individuals [60].

ERAD system provides an effective cleaning of misfolded proteins accumulated in the ER, either because of mutation, incorrect translation or even inability to form the

correct protein complexes. Thus, *de novo* translated proteins in ER are glycosylated in order to control and manage the folding maturation of the protein. This glycosylation is supported by an enriched-mannose N-glycan with 3 final residues of glucose. Two of those glucose residues are de-glycosylated by glycosidase I and II, resulting in a new glycoprotein recognized by the CNX/CRT system. CNX/CRT associates with PDI to promote the oxidizing environment needed to the correct protein folding. This system is highly effective if the misfolded error has its origin from posttranslational stages. However, if this system is badly-succeeded, α-mannosidases and ER degradation enhancer mannosidase (EDEM) cleaves the terminal mannose residues from the glycoprotein allowing the recognition by other two ER resident enzymes: osteosarcoma 9 and XTP3-transactivated gene, both containing the mannose-6-phosphate receptor-like domain, necessary for the mannose residues recognition. As the proteasome machinery is in the cytosol, those misfolded recognized proteins need to translocate from ER lumen to the cytosol. The mechanisms governing this second ERAD step (retrotranslocation) are still not completely fully disclosed and their comprehension is an extremely challenging scientific field. Once in the cytosol, misfolded proteins are ubiquitinated by the E1-E2-E3 ubiquitin system (activation-, conjugation- and ligation-enzymes, respectively) and enter into the proteasome, in order to be degraded (reviewed in [54]).

Finally, autophagy is another process by which stressed cells are able to clear the high levels of misfolded proteins. Despite the established relation between ER stress and autophagosome formation, the molecular mechanism behind these two events is still under discussion. In an exploratory study to understand which of ER stress-sensor could be involved in the formation of autophagossomes, the process was shown to be dependent on the IRE1-JNK pathway, as IRE1-deficient cell were unable to form autophagosomes [61]. However, in a Huntington *in vitro* disease model (polyglutamine aggregation) Kouroku and his team observed that autophagosomes formation was rather dependent of PERK/eIF2 pathway [62].

These cytoprotective mechanisms (UPR, refolding, ERAD and autophagy) should be able to recover the ER and cell homeostasis. However, in pathologic/inflammatory conditions, the persistent ER stress can switch the cytoprotective functions of UPR and autophagy into cell death promoting mechanisms [47].

3.3 ER stress-induced apoptosis

Apoptosis, or programmed cell death, is the ultimately consequence when cells were not able to restore ER homeostasis. In those conditions, IRE1 and PERK activate two parallel pathways: JNK and CHOP apoptotic pathways, respectively [47]. The prolonged ER stress state is reflected by the overexpression of IRE1 which is intimately related to the increased levels of CHOP [63]. CHOP blocks anti-apoptotic molecules expression such as Bcl-2 family proteins and upregulates GADD34 and TRB3 [64]. In parallel, IRE1 associates with TRAF2 activating ASK1 that in turn triggers JNK-dependent apoptotic processes: the phosphorylation of BH3-only members of Bcl-2, such as Bim (a pro-apoptotic molecule), induces its migration from the cytoskeleton to the ER membrane and phosphorylation of Bcl-2 protein leads to its suppression [47]. This 2 different action of ASK1 phosphorylation converges to the cell apoptotic fate. Bcl-2 family proteins Bax and Bak are also associated with ER-stress apoptosis. When "free" Bax and Bak levels increase in cytosol, they migrate into the mitochondrial outer membrane, oligomerize and create mitochondrial pores, allowing cytochrome c to leak over the cytosol [65]. Once released, cytochrome c activates the caspase cascade, culminating in cell apoptosis. Bak and Bax were first linked to ER stress-induced apoptosis when Wei and colleagues induce apoptosis (Fas-independent) in $Bak^{-/-}$ and $Bax^{-/-}$ mice and observed a higher survival rate, compared to the other genotypes [66].

As mentioned before, one of the stimuli that can trigger ER stress is the alteration of Ca^{2+} levels. In this case, an UPR-independent pathway is additionally activated: activated calpain cleaves procaspase-12, releasing caspase-12 to migrate towards the cytosol and activate the caspase cascade [66].

3.4 ER stress in human diseases

In recent years, interest in ER stress mechanisms greatly increased due to its association with various diseases and pathological states. The most well-known are neurodegenerative pathologies such as Alzheimer, Huntington, Parkinson and Prion diseases. These diseases are characterized by the accumulation of misfolded proteins and an impaired protein degradation system, affecting different cell signalling pathways which can result on suppression of neuronal communication or even cell death. In Alzheimer

disease, IRE1 was found to be inhibited by mutant protein S from secretase complex, leading to an impaired UPR response (reviewed in [67]). However some studies showed increased levels of PERK in brain of Alzheimer's patients, indicating that ER stress-apoptotic pathway could be activated (reviewed in [67]). In Parkinson's disease the levels of UPR chaperones and phosphorylated IRE1 and PERK, as well as CHOP, clarified the involvement of the UPR in this pathology [68]. In turn, Huntington disease present a particular feature caused by a mutation in chromosome 4, leading to expanded polyglutamine repeats in various proteins. ER stress is triggered by a hampering and impairment of proteasomal system, leading to an accumulation of these proteins in ER lumen. Also high levels of CHOP and GRP78 were found in Huntington cells and ASK1 (from IRE1-pathway) was shown to be essential to polyglutamine-induced ER-mediated cell death [69]. Additionally, ER stress is also associated with proinflammatory chronic processes, such as type 2 diabetes, atherosclerosis, intestinal bowel disease and cancer. Nevertheless ER stress-induced inflammation has not a linear effect on all cells and tissues, and the final output will depend on the targeted cell and its microenvironment. While in the majority of the referred inflammatory pathologies ER stress is a deleterious process culminating in cell death, in cancer it can either act as a pro-survival mechanism or be involved in tumor cell apoptosis and immunogenic signals (reviewed in [70]).

3.5 Paradoxical role of ER stress in cancer

Tumor cells are surrounded by a very particular microenvironment characterized by adverse conditions to cell survival, such as hypoxia, nutrient deprivation, low pH and high levels of ROS. In such extreme conditions, protein synthesis is highly affected, either because of the insufficient glycosylation, the unfavourable conditions to the establishment of disulphide bonds, or the proteins transformation by ROS interaction. As a result, the cell enter in a prolonged ER stress and UPR mechanism is not sufficient to restore homeostasis [71]. Several studies reported higher levels of ER stress in cancer cells, comparing to the healthy tissues, as for example in colorectal, lung and mammary carcinoma (*48-50*). In a study with colon cancer as a model, 98% of biopsies from cancer patients stained positive to GRP78 whereas only 48% of samples from healthy tissues stain positive [55]. Moreover, cancer lung cells have also been shown to present higher levels of ER stress

(about 3-fold) when compared to healthy cells [57]. These results, among others, contributed to the understanding of the close relation between tumor cells and high and chronic levels of ER stress. Despite of the clear evidences pointing to elevated ER stress in solid tumors, its outcome in cell death or survival remains inconclusive. As described before, prolonged ER stress leads to the activation of pro- apoptotic pathways of the UPR. Therefore, its most logic role in cancer would be as an anti-tumoral process. However, increasing evidences point that ER stress may be central in the modulation of several pro-tumoral mechanisms [71]. UPR activation was shown to be essential to the tumor survival under hypoxic conditions [51,72,73]. The higher apoptosis rate of XBP1-deficient cells under hypoxic conditions indicated that XBP1-deficient cells are more sensitive than the wild-type ones [72]. Also PERK-deficient tumors were more sensitive to hypoxia as they grew less than the PERK-positive tumors [73]. These studies converged to the hypothesis that UPR activation is fundamental to cell survival under hypoxic conditions; however, the mechanisms behind those associations aren't yet clear.

In addition to those tumor cell-intrinsic mechanisms, ER stress is also implicated in extracellular actions, all associated with pro-survival events of the tumor. It is well established that under hypoxic conditions tumor cells secrete VEGF though a HIF-1α-dependent mechanism in order to promote vascularisation. However, some studies showed that the upregulation of VEGF was HIF-1 independent in some cellular models. On those cases, ER stress plays an important role as the UPR mediator IRE1 is the key factor on the upregulation of VEGF [74]. Also PERK has some influence in angiogenesis regulation, as showed in experiments where $PERK^{-/-}$ mice developed smaller tumors than PERK-positive mice. Moreover, the formation of microvessels was analysed *in vivo* and the authors observed about 16-fold more vessels counted by section of tissue on $PERK^{+/+}$ mice, concluding that PERK activation is important to the vascularization of the tumor [75].

As an additional mechanism that links UPR to tumor survival, chaperones such as GRP78, were shown to protect tumor cell against cytotoxic T cells mediated lysis [76]. This finding was demonstrated in an elegant experiment where GRP78 silenced cells were injected in mice with fibrosarcoma, being the progression and incidence of tumors monitored along the time. Mice with suppressed GRP78 showed a lower incidence and progression of tumors, and the regression of the initially formed tumors pointed to some anti-tumoral attack by CTL [76].

Inflammation is also a characteristic of tumor microenvironment and is influenced by ER stress in tumor and surrounding cells. Inflammatory mediators present in tumor microenvironment induce tumor-promoting cytokines, such as IL-11, IL-1β, IL-6, IL-23 and TNF that can act as autocrine and paracrine survival, growth and metastatic factors. This is view as a "private" signalling between tumor cells, as they upregulate, for example the IL6 receptor, exerting its proinflammatory and anti-apoptotic action more prominently on cancer cells (reviewed in [70]). High levels of TNF-α cause ROS accumulation, which in turn lead to the activation of UPR signalling (to rescue the oxidized proteins). Production of ROS and protein folding are closely linked events. Uncontrolled production of ROS can directly or indirectly affect ER homeostasis and perturbations in protein folding can cause alterations in cellular redox status increasing the generation of mitochondrial ROS. High levels of cytokines, especially TNF-α, in addition to ROS induction, are also responsible for the direct activation of UPR-sensors, by the NF-kB signalling pathway (reviewed in [70]). IRE1α associates with TRAF2 and together mediate IkB phosphorylation resulting in NF-kB activation. Also ATF6/AKT and JNK have been associated to NF-kB activation and consequent inflammatory gene upregulation (reviewed in [71]). Therefore, tumor proinflammatory microenvironment induces ER stress which in turn enhances inflammatory mediators expression, among tumor and immune cells. ER stress/inflammation/tumor cells association suggested that tumor cells under ER stress could transmit stressing signals to neighbour cells modulating their activity and functions.

Recently a new body of data highlight the role of tumor UPR as a critical modulator of antitumor immunity through transmissible extrinsic effects on infiltrating immune cells, a mechanism denominated Transmissible Endoplasmic Reticulum Stress (TERS) (Table 2). Macrophages cultured in conditioned medium from ER-stressed tumor cells were shown to strongly develop ER stress [77]. In posterior studies the same group evidenced that DC cultured in the same ER-stressed tumor cells conditioned media present clear evidences of ER stress with high levels of GRP78 and arginase 1 expression [42]. ER stress in DCs impairs the translation and posttranslational mechanisms that will result in the incapacity to properly fold MHC I and CD80, CD86, CD40 and other co-stimulatory molecules, essential for antigen presentation. In fact, *in vivo* experiments showed that TERS-imprinted DCs were inefficient in priming $CD8^+$ T lymphocytes [42].

Apart from the impaired protein translation, the activation of UPR pathways in DC will also lead to a pro-tumorigenic phenotype, mainly through secretion of cytokines and growth factors. ER stress and Toll-like receptor stimulation induced, in myeloid DCs, an upregulation on p19 subunit of *IL23*, as well as an increased expression of IL-23 and several chemokines [42,43]. Additionally, ER-primed DCs overexpress immunosuppressive molecules such as B7 homolog 1 (B7-H1) [42] that are well known to inhibit $CD8^+$ T cell proliferation and to cause suppression of antigen cross-presentation [78]. In fact, experiments in which tumor-imprinted ER stress DCs were cultured with $CD8^+$ T cells resulted in a marked alteration of their transcriptional profile: upregulation of *IL10, TNFα and FOXP3*, as well as the downregulation of co-stimulatory molecule, *CD28* [42]. Such profile is comparable to Treg phenotypic profile, and indicates that ER stressed primed DCs have the capacity to polarize $CD8^+$ T cells towards a more regulatory profile (Table 2).

Overall, these data suggest that tumor secretome may be the key behind the tumor cells "Transmissible" ER stress (TERS). However, the precise nature of stimuli that trigger ER stress in tumor cells and the vehicle by which ER stress is transmitted is still unidentified. In the present work we hypothesized that this transmission could be accomplished by tumor-derived exosomes. Exosomes are involved in multiple tumor extrinsic signalling processes, namely in the immunosuppression, in the extracellular matrix, vascular and stromal cells transformation and also in drug interference (for more details see [79]). Recently, new data have highlighted the important role of tumor-derived exosomes on the modulation of surrounding cells in order to promote and benefit the tumorigenesis process. Melo and her colleagues have reported and described the capacity of breast cancer-derived exosomes to reprogram non-malignant cells into tumorigenic cells, driven by exosomal microRNAs [80]. The study of exosomes as a possible vehicle to communication, re-programmation and transport of several factors is still on the beginning however seems very promising.

Table 2 - Incident studies reporting an association between tumor-induced ER stress and changes in protein expression profile.

Target cell	Cell-derived CM	ER stress inducer	Results	Ref.
Mo	Prostate cancer	Thapsigargin		
	Lewis lung carcinoma and metastic melanoma	Thapsigargin/ lacking of glucose	Upregulation of *GRP78*, *GADD34* and *CHOP*; overexpression of IL-6, IL-23p19	[77]
BMDM	Prostate cancer	Thapsigargin	The same results as the previous the ones with Mo; overexpression of TNF-α, MIP-1α and MIP-1β	[77]
BMDC	Lewis lung carcinoma, metastic melanoma and prostate cancer	Thapsigargin	Upregulation of *GRP78* and downstream of *XBP1* and *CHOP*; Upregulation the transcription of *IL6*, *IL23p19* and *TNFα*; Oversecretion of IL-6, IL-23, TNF-α, TGF-β, MIP-1α, MIP-1β and MCP-1. Overexpression of arginase 1 Overexpression of PD-L1 Decreased levels of *CD28*, increased levels of *Lag3*, *IL10* and *TNFα* and *FOXP3* on T cells	[42]

Aim of the study

Tumor microenvironment provides particular conditions that are prone to favour tumor cells survival and proliferation even under attack by immune cells. A wide spectrum of immunoescape mechanisms have been reported and studied for years, however the role of tumor-induced ER stress in immune cells is not fully disclosed. Recent studies proposed that tumor stressed cells were able to transmit ER stress to surrounding cells including DCs, subverting their maturation process and their immunostimulatory abilities. In fact until now, only two studies reported tumor-induced ER stress in myeloid cells, remaining the mechanism by which such ER stress is transmitted completely unknown.

Thus, the main objectives of this work are:

1. To evaluate the capacity of tumor microenvironment stressors, such as hypoxia, acidic medium and glucose deprivation to induce ER stress in a breast cancer luminal A cell line (T-47D cells)
2. To evaluate the capacity of conditioned medium from tumor cells to induce ER stress in myeloid dendritic cells.
3. To assess the effects of this transmitted ER stress on DCs phenotype and functions
4. To study the factors/mechanisms responsible for the transmission of ER stress by the tumor cells

Methods and materials

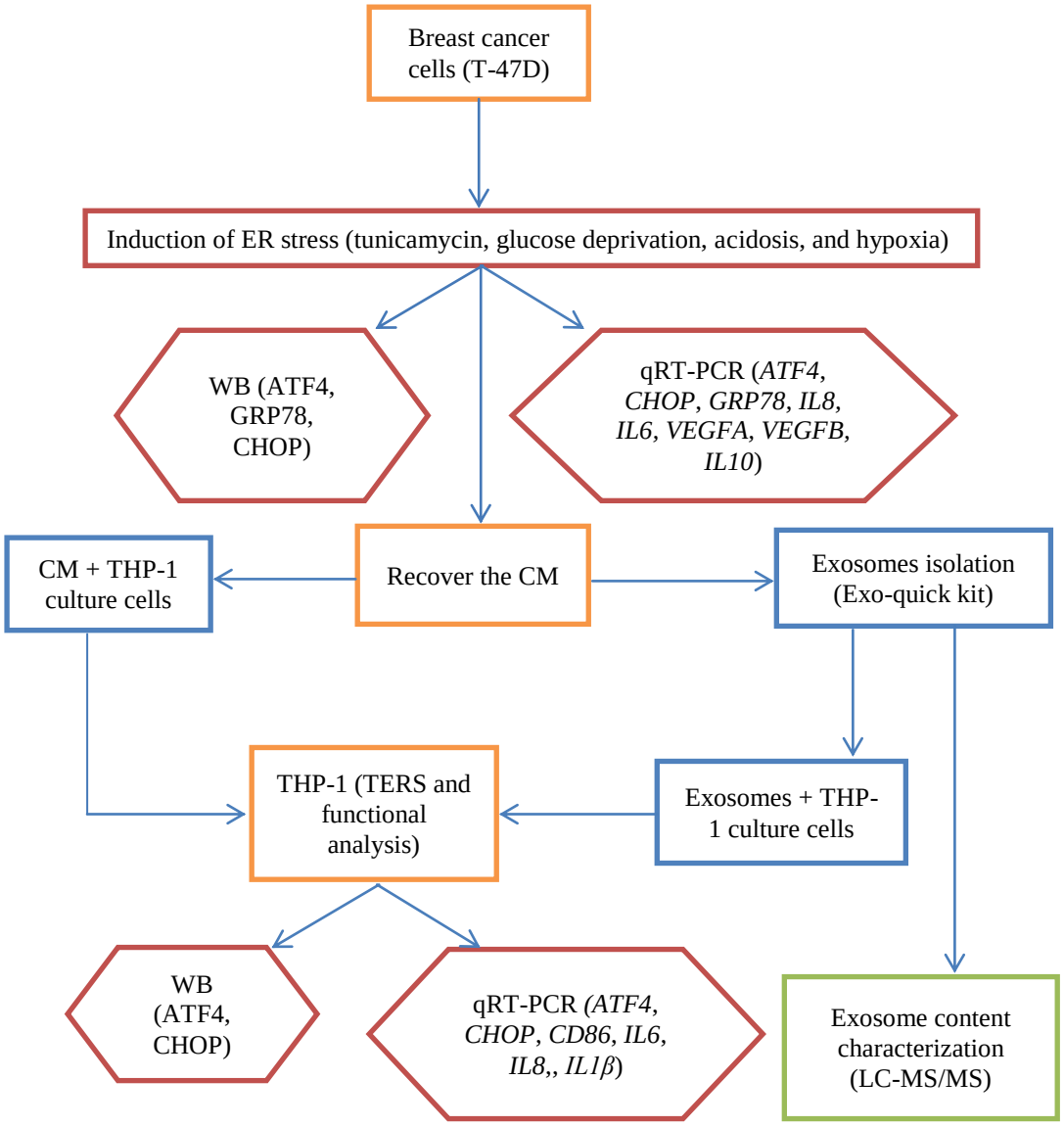


Figure 6 – Schematic representation of the general methodology approaches conducted in this project.

1. Cell Culture

T-47D human breast cancer adherent cells (ATCC HTB133, American Type Culture Collection, Manassas, VA) were routinely seeded and maintained in T-75 culture flasks in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA), 25 mM glucose, 10 mM HEPES, 100 µg/mL streptomycin, 1mM sodium pyruvate and 100 U/mL penicillin (designated hereinafter by RPMI 10% FBS); Cell were incubated at 37ºC in humid atmosphere with 5% carbon dioxide. Passaging was performed when cells reached confluence level about 80%. For different experimental approaches, cells were incubated and treated as described above:

1) Induction of ER stress in breast cancer cells: T-47D cells were plated in 100mm plastic dishes and/or 6-well-plates at the respective seeding density and incubated with the different medium composition described in Table 3. All the different growth conditions were maintained for 4h and 24h before RNA/protein extraction; cells were grown until 60% confluence.

Table 3 – Description of the cell culture features and treatments associated to the induction of ER stress in T-47D cells

T-47D Stimuli	Seeding density		Growth medium composition
	WB	qRT-PCR	
Tunicamycin	1.0 x 10^6 cell (0.3 x 10^6 for T-47D cell metabolism assay)	0.30 x 10^6 cell	RPMI 10% FBS + tunicamycin (Sigma) 10 µg/mL
Glucose deprivation			RPMI 10% FBS without glucose
Lactic Acidosis			RPMI 10% FBS + lactic acid (pH=6)
Glucose deprivation + lactic acidosis			RPMI 10% FBS without glucose + lactic acid (pH=6)
Phenantroline			RPMI 10% FBS + phenantroline (Sigma) 15µmol/mL
Cobalt chloride ($CoCl_2$)			RPMI 10% FBS + $CoCl_2$ (Sigma Aldrich)
Hydrochloric acid (HCl)			RPMI 10% FBS + HCl (Sigma) (pH=6)
Glucose deprivation + HCl			RPMI 10% FBS without glucose + HCl (pH=6)

2) Conditioned medium (CM) production: T-47D-derived CM was obtained by culturing T-47D cells in T-75 flasks until 60% confluence. Then ER stress was induced either by tunicamycin or glucose deprivation (Table 4). Tunicamycin was washed after 4h of incubation and T-47D cells were supplemented with fresh

medium and grown for 24h. After 24h CM was recovered and centrifuged at 5000 g for 10 min to remove cell debris.

3) Exosomes production: T-47D-derived exosomes were obtained by culturing T-47D cells in the same conditions as CM production (point 2) except that RPMI medium was supplemented with 10% exosome-free FBS (Table 4);

Table 4 - Description of the cell culture features and treatments associated to the exosome-producing T-47D cells.

T-47D Stimuli	Seeding density	Growth medium composition
Tunicamycin	2.1 x 10^6 cell	RPMI 10% FBS + tunicamycin 10 µg/mL
Glucose deprivation		RPMI 10% FBS without glucose

4) T-47D ER stress and glucose metabolism experiment: T-47D cells were grown in 60 mm plates and/or 6-well-plates (for protein and RNA extraction respectively) for 24h on the respective treatment (tunicamycin, glucose deprivation, glucose deprivation + lactate, lactate, glucose deprivation + hydrochloride acid (HCl) and hydrochloride acid) as described in Table 3.

5) THP-1 culture and incubation with CM and/or T-47D-isolated exosomes: THP-1 suspension cells were routinely maintained with RPMI 10% FBS. For TERS assessment, THP-1 cells were left to grow for 48h in 6-well-plate (with initial seeding density of 0.8 x 10^6 cell/well) and incubated with 3 mL of CM and/or T-47D-isolated exosomes. For maturation status and cytokine analysis THP-1 cells were treated with 1µg/mL of lipopolysaccharide one hour prior to exosomes incubation.

2. Evaluation of cell viability

Resazurin reagent was used to assess cell viability, using different concentrations of tunicamycin. Cells were seeded in 96-well-plate and exposed to different tunicamycin concentrations (1, 2, 5, 10 µg/mL) for 24h. Occurred that period, resazurin (Sigma Chemical Co. St. Louis, MO, USA) was added for 4h with a final concentration of 50 µM, and absorbance was read at 570 and 600 nm in Multiskan Go spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3. Protein extraction and quantification

To obtain whole cell lysates, T-47D and THP-1 cells were washed in ice-cold PBS and harvested in RIPA lysis buffer (50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA) freshly supplemented with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany) and 1 mM DTT. The insoluble cell debris was then removed by centrifugation at 14000 g at 4 ˚C for 10 min. Supernatant was collected and used as total cell lysate. Protein concentration was determined using the bicinchoninic acid method (Sigma Chemical Co, St. Louis, MO, USA) [81]. To obtain exosomal protein quantification, purified eluate-containing exosomes was harvested in RIPA lysis buffer in non-reducing conditions (50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA), freshly supplemented with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). The insoluble debris was then removed by centrifugation at 4ºC at 14000 g for 10 min. Supernatant was collected and used as total exosome lysate. Protein concentration was determined using the bicinchoninic acid method (Sigma Chemical Co, St. Louis, MO, USA) [81].

4. Western-Blot

For CHOP, ATF4, GRP78, CD81 and CD63 detection it was added denaturing buffer (0.125 mM Tris pH 6.8; 2% (w/v) SDS; 100 mM DTT; 10% (v/v) glycerol and bromophenol blue) to each supernatant (1:1) and the samples were boiled for 5 min at 95ºC. Then 20-30 µg of total lysate was applied to a SDS-PAGE 12% (v/v) bisacrilamide/acrylamide gel. Proteins were separated by eletrophoresis (140 V, 90 minutes) and transferred to polyvinylidene difluoride (PVDF) membranes (Milipore, Bedfore, MA, USA). Non-specific binding was blocked with 5% (w/v) of skimmed milk in TBS-T (100 mM Tris pH 8.0, 1.5 mM NaCl and 0.1% Tween-20) during 1h at room temperature and under soft and continuous stirring. To detection of ER stress markers, membranes were incubated overnight with monoclonal antibody rabbit anti-ATF4 (Cell signalling Technology), mouse -CHOP (Cell Signalling Technology), rat -GRP78 (Biolegend, San Diego, CA, USA), mouse/rat –CD81 (Biolegend, San Diego, CA, USA) and mouse –CD63 (Biolegend, San Diego, CA, USA) diluted by 1:1000 factor each one;

The membranes were then washed three times to remove unbound antibodies, for 10 min each, in TBS-T, followed by a secondary antibody incubation anti-rat, anti-mouse or anti-rabbit associated to the alkaline phosphatase, for 1h room temperature. Finally membranes were exposed to ECF (GE Healthcare, Chalfont St. Giles, UK) reagent and immunocomplexes were detected by scanning the membrane for blue excited fluorescence on the Typhon (GE Healthcare, Chalfont St. Giles, UK) and analysed using the software TL120 v2009 (TotalLab Ltd, Durham, USA). As loading control, membranes were stripped and incubated with anti-tubulin antibody.

5. RNA extraction

For RNA extraction, T-47D and THP-1 cells were cultured as indicated previously in Table 3 for 4 or 24h. Total RNA was isolated from cells adding 1 mL of NZYol reagent (NZYTech, Lisbon, Portugal) to each well. After pipetting several times the cell lysate, 200 µL of chloroform was added and the sample was shacked vigorously, incubated for 2 min at room temperature and centrifuged for 12,000 g for 15 min at 4ºC. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 500 µL of isopropanol for 15 min at room temperature. Then the solution was centrifuged at 12,000 g for 10 min at 4ºC and the pellet was washed with 1 mL of ice-cold 75% (v/v) ethanol and re-suspended in 40 µL 60ºC heated Storage Solution RNAse free (Ambion, Foster City, CA, USA). The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA). Quality was monitored for absence of genomic DNA, protein or guanidine isotiocianate contamination through assessment of 260/280 and 260/230 ratios.

6. qRT-PCR

For the synthesis of cDNA the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) was used according to manufacturer instruction. Briefly, 2 µL of random primers and the necessary volume of RNAse-free water to complete 15 µL were added to 1 µg of total RNA. After this, 10 µL of NZYRT 2x Master Mix, 2 µL of NZYRT Enzyme Mix and 20 µL of DEPC-treated H_2O were added to each sample. A protocol for cDNA synthesis was run on all samples (10 min at 25ºC, 30 min at 50ºC, 5 min at 85ºC and then chilled at 4ºC). Quantitative Reverse Transcription Polychain Reaction (qRT-PCR) was

performed in 20 µL reaction volume: cDNA (25 ng), 10 µL 2x SensiMix™ SYBR® (Bioline, MA, USA), 2 µL of each primer (250 nM) and 3.5 µL RNAse free H_2O. A protocol for cDNA amplification was run on all samples (10 min at 95ºC and 40 cycles of: 15 sec at 95ºC, 15 sec at 55ºC and 20 sec at 72ºC). qRT-PCR reactions were run in duplicate for each sample on a Bio-Rad CFX Connect™ Real Time PCR detection system. Primers were designed using Beacon Designer software v8.0, from Premier Biosoft International. Primer sequences used are given in Table 5. On RT-PCR plate there was a non-template control for each pair of primers analysed. For determination of specific efficiencies, a 4 points dilution series of control sample for each pair of primers was run on each experiment. After amplification, a threshold was set for each gene and Ct-values were calculated for all samples. Gene expression changes were analysed using the built-in Bio-Rad CFX Manager 3.1 from Bio-Rad.

Table 5 – Primer sequences used for qRT-PCR analysis.

Gene Name	Forward primer	Reverse Primer
GADPH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC
HPRT1	TGACACTGGCAAAACAATG	GGCTTATATCCAACACTTCG
TATA	TTCCACTCACAGACTCTC	ACAATCCCAGAACTCTCC
IL1β	GCTTGGTGATGTCTGGTC	GCTGTAGAGTGGGCTTATC
IL6	ACCTCAGATTGTTGTTGT	GTCCTAACGCTCATACTT
IL8	CTTTCAGAGACAGCAGAG	CTAAGTTCTTTAGCACTCC
CD83	ATTGAGTCATTATCCTTGCTAT	GCTTCTTGGTAACCTTCTT
CD86	GAACCTAAGAAGATGAGT	TCCAGAATACAGAAGATG
CHOP	CTGGAAGCCTGGTATGAG	GGTCAAGAGTGGTGAAGAT
ATF4	AGATAGGAAGCCAGACTA	CTCATACAGATGCCACTA
GRP78	TTACTTAGATTGTGTTCCT	TCTCTATCTCTTGACCTT
VEGFA	TTACTCTCACCTGCTTCT	CTGCTTCTTCCAACAATG
CXCL14	TTCCAAGATCCTGTGATG	TATTGAACCTGTGAACCTT
VEGFB	ATCTACTGAGTGACCTTG	TTCTGTTCCCTCTATGTG

7. Exosomes isolation and purification

T-47D cells were grown in aforementioned conditions (Table 4, section *1) Cell Culture*), in which bovine exosomes have been depleted from media. For cell debris removal, initial volume of 12 mL was centrifuged at 370 g for 10 min at 4ºC. The supernatant was centrifuged again at 16,000 g for 30 min at 4ºC. Then, exosomes were isolated from conditioned medium with Exo-spin™ kit (Cell Guidance Systems, Carlsbad, CA, USA). Briefly, to precipitate the exosome containing fraction, 5 mL of Buffer A from

was added to 10 mL of supernatant. Gentle inversion was performed to ensure proper mixing which was then incubated overnight at 4ºC. Following a centrifuge of 20,000 g for 1h, the supernatant was carefully removed and discarded. The left exosome containing pellet was re-suspended in 100 µL 1x PBS. Finally, to purify the exosome-containing solution, the re-suspended exosomes was eluted in spin column (provided with the kit). Ultracentrifugation was also performed in order to remove exosomes from conditioned medium and assay the effect of conditioned medium depleted from T-47D-exosomes, following Thèry et al protocol [82].

8. Transmission Electron Microscopy (TEM) Analysis

Exosomes were observed by Transmission Electron Microscopy (TEM). For TEM analysis, 20 mL of bovine exosomes depleted conditioned medium (from around 20 x 10^6 T-47D cells) was recovered and T-47D-exosomes were isolated as previously described purified exosomes pellet was firstly fixed and then contrasted and embed on electron microscopy grids following Thèry et al protocol [82]**.** Briefly, 20 µL of paraformaldehyde (PFA) 4% was added to exosome-containing eluate (20 µL) and 5 µl deposited onto Formvar-carbon coated EM grids. Exosome-coated vesicle were washed with 100 µL of 1x PBS and after 50 µL of 1% glutaraldehyde for 5 minutes. The grids were then washed 8 times with distilled water with stand periods of 2 minutes. Following the fixation process, samples were contrasted and embedded: 50 µL of uranyl-oxalate pH 7 were transferred to the grids and incubated for 5 minutes; then grids were embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose (100 µL/ 900 µL). Grids were then incubated with 50 µL of methyl cellulose-UA for 10 minutes, on ice. Finally, the grids were dried and observed under the electron microscopy at 80kV.

9. Statistical analysis

The results are presented as mean ± S.D., and the statistic difference between two groups was determined by the two-sided unpaired Student's t test. The tests were performed using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). Statistically significant values are as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

Results and Discussion

Chapter I - Unravelling the possible mechanisms of Transmissible Endoplasmic Reticulum Stress phenomena

In order to evaluate our hypothesis that ER stressed cancer cells may transmit ER stress factors to other cells (transmissible ER stress (TERS)) modulating that way their microenvironment, we performed several experiments to: 1) optimize a pharmacological and a physiological ER stress-induced model; 2) evaluate TERS in monocytes; 3) assess monocytes modulation by TERS; 4) elucidate the vehicle by which TERS occurs. Initially we optimized the effect of either tunicamycin and glucose deprivation on cells, establishing an ER stressed cancer cell model. Then we evaluate the transmission of ER stress from cancer cells to monocytes, either by using total conditioned medium or isolated exosomes. Finally we assessed the effects of ER stress-derived exosomes on monocytes activation status and inflammatory phenotype.

1. High concentrations of tunicamycin do not induce relevant cytotoxicity in T-47D cells

Tunicamycin (TUN) is commonly used as a positive inducer of ER stress, leading to UPR activation in several cell types. Briefly, tunicamycin is an antibiotic that inhibits protein N-glycosylation resulting in the accumulation of misfolded proteins in the ER lumen [83]. As a result, ER membrane transducers, IRE1, ATF6 and PERK are activated and trigger the three UPR-branches that culminate in the overexpression/phosphorylation of some ER stress protein markers such as GRP78, CHOP, ATF4, p-eIF2α, among others [63,83–85]. To prevent death events due to over dosage we assessed in a first stage of our experiments, the impact of tunicamycin on breast cancer cells (T-47D) viability using resazurin. Resazurin is a non-toxic blue dye that is used to detect NADH, released by metabolic active cells. Through a redox reaction, resazurin turns into a pink oxidized compound, resofurin, possible to quantify by spectrophotometry

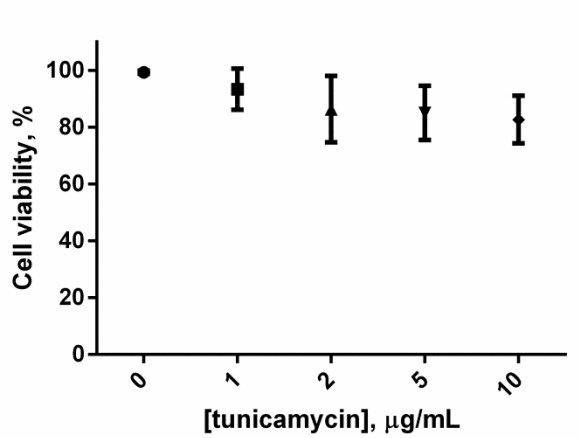


Figure 7 – Effect of different concentrations of tunicamycin on T-47D cell viability. $3x10^4$ T-47D cells were treated with different concentrations (1, 2, 5 and 10 µg/mL) of tunicamycin for 24h. 50 µM resazurin were added in the last 4h. The absorbance was then read at 570 and 600 nm in a standard spectrophotometer.

From our results, T-47D cells showed to be resistant to high concentration of tunicamycin. Even the highest concentration tested (10 µg/mL) just resulted in a slight decrease of cell viability (Figure 7). In fact, this slight decrease may even result from a diminished mitotic process caused by tunicamycin rather than from cell death. Thus, to maximize cells response to the drug, we decided to use an elevated concentration of tunicamycin.

2. Tunicamycin, glucose deprivation, acidosis and phenantroline induce ER stress in T-47D breast cancer cells

As previously described, UPR in tumor cells is effectively activated by several stimuli such as hypoxia, nutrient deprivation and acidosis among others. To mimic these microenvironment factors T-47D cells were incubated for 24h without glucose (-GLU), in lactic acidic medium (ACID), with the chemical hypoxia inducers - cobalt chloride and phenantroline ($CoCl_2$ or Phe) or with a glucose free acidic medium (-GLU+ACID) (Figure 8). Induction of ER stress was then monitored by analysis of transcription and expression of the UPR-related proteins ATF4, CHOP and GRP78.

Figure 8 – Brief scheme of cell treatments. Cells were stimulated for 4/24h for each stimulus (tunicamycin, acidosis, glucose deprivation, glucose deprivation and acidosis, phenantroline and cobalt chloride) and then protein and/or RNA were extracted.

As expected, tunicamycin induced the overexpression of UPR-related proteins, namely CHOP, GRP78 and ATF4. Moreover, we observed that tunicamycin effect was enhanced at longer incubation times (24h). Hereinafter, tunicamycin was undertaken as our experimental positive control for ER stress induction. Consistent with previous reports [85,86], we observed that glucose deprivation effectively induced ER stress in T-47D cells (Figure 9). Glucose starvation early activates the UPR as shown by the strong increase of ATF4 levels at 4h post deprivation. In contrast to tunicamycin, these high ATF4 levels were maintained over 24h and resulted in a strong induction of CHOP protein. Concerning lactic acidosis (ACID) stimulus, it is clear the absence of effective UPR activation, as visible by the very low levels of CHOP, GRP78 and ATF4 for both 4 and 24h. Lactic acidosis has been frequently referred to as a tumor microenvironment ER stress inducer, as lactate is an abundant product present in the intercellular space of the solid tumors; however little is known relatively to its role in the UPR activation, and relatively few studies have reported its relevance. High acidosis (pH 7.0-6.0) reduces ER Ca^{2+} ATPase activity, explaining the induction of ER stress, in these conditions [87]. One of the few studies linking ER stress and acidosis reported that astrocytes cultured at pH 6.0 overexpress GRP78 [88]; however tumor microenvironment pH ranges between 7.0 and 6.5 [89], and this milder acidosis seemed to not induce ER stress in T47-D cells (Figure 9).

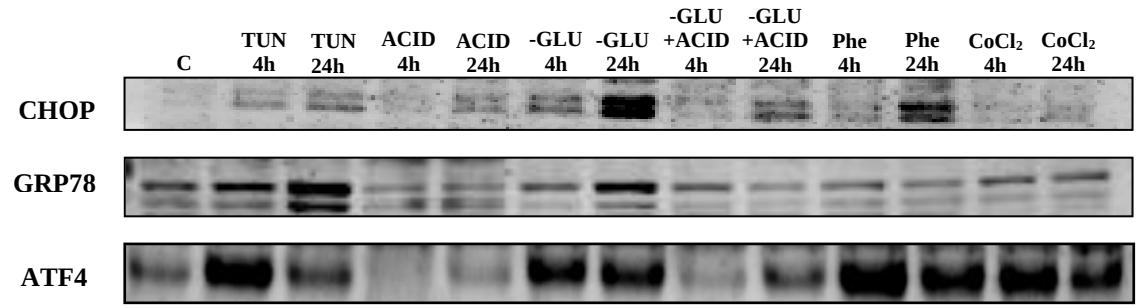
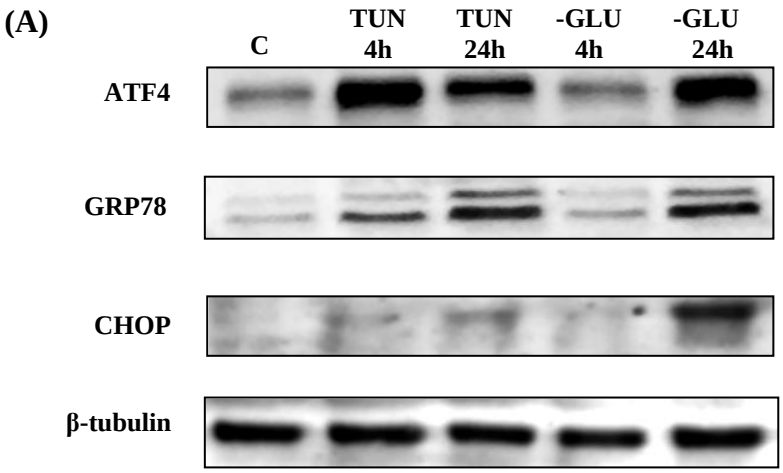


Figure 9 - Effect of tumor environment factors (or mimics) and tunicamycin on the levels of CHOP GRP78 and ATF4 proteins. T-47D cells were stimulated for 4/24h with each stimulus (tunicamycin - TUN; acidosis – ACID; glucose starvation - -GLU; glucose starvation and acidosis - -GLU+ACID; phenantroline – Phe; and cobalt chloride – $CoCl_2$. After the indicated times, total cell lysates were prepared and UPR markers analysed by Western blotting.

Surprisingly, T-47D cells grown in medium without glucose combined with lactic acidosis (25 mM lactic acid, pH 6.5) seemed to present lower levels of ER stress compared

to glucose deprivation itself (Figure 9). The lower levels of ATF4, GRP78 and CHOP, observed when lactic acid was added to deprived glucose medium suggested us that lactate could be used as an alternative energy source, which can protect T-47D towards the ER-stress induced by lack of glucose. This hypothesis was studied and discussed later, in chapter II of this section - *Lactic acidosis mitigates glucose deprivation induced-ER stress in T-47D cells.*

After this initial screening for identification of effective ER stress modulators, tunicamycin as a positive control and glucose deprivation as physiological stress inducer were chosen for the design of subsequent experiments. We began by performing a more detailed assessment of the effects of tunicamycin and glucose deprivation on the expression and transcription of UPR effectors. To do so, we incubated T-47D cells with tunicamycin or glucose deprived medium and extracted protein and mRNA at 4 and 24h.



(B)

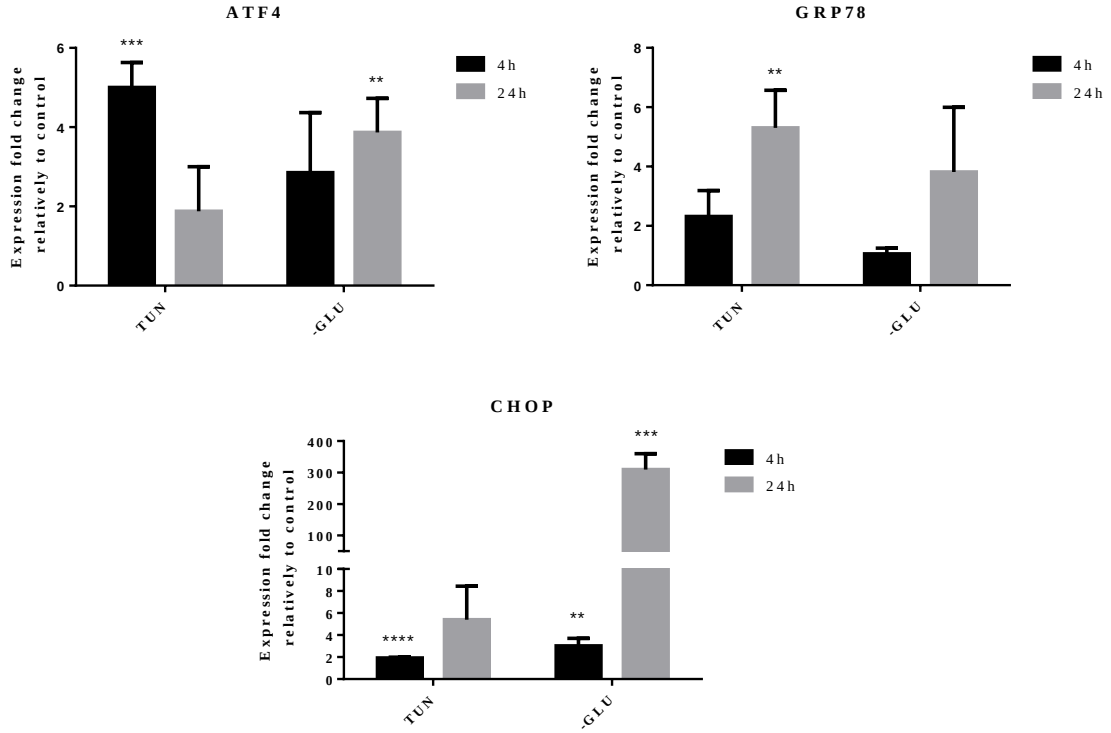


Figure 10 - Effects of tunicamycin and glucose deprivation on the levels of ATF4, CHOP and GRP78 proteins on cancer cells. (A) T-47D cells were stimulated for 4 or 24h with tunicamycin (TUN) and glucose deprived medium (-GLU). Then total protein extracts were prepared and ER stress evaluated through analysis of ATF4, CHOP and GRP78 protein levels by Western blotting **(B)** The results represent at least 3 independent experiments and were expressed as fold changes relatively to control (untreated cells). *p<0.5; **p<0.01; ***p<0.001; ****p<0.0001

Western blot analysis revealed that ATF4 protein expression highly increases after tunicamycin treatment or glucose deprivation (Figure 10A). It is worth to note that ATF4 protein expression is promptly increased for both stimuli; however high ATF4 protein levels were only sustained for glucose deprivation, as in tunicamycin-treated cells ATF4 levels substantially diminished at 24h. ATF4 is a bZIP transcription regulator, controlled by eIF2α phosphorylation that, in turn, is activated by one of 4 stress sensors, namely ER stress-related PERK, amino acid deprivation sensor GCN2, infection with double stranded RNA viruses (PKR) and heme deficiency (HRI) [49,90]. ATF4 has a relevant role in tumor survival, mainly due to its functions as UPR effector protein (upregulating chaperones such as GRP78, inhibiting generalized protein synthesis, and inducing the CHOP/GADD34 apoptotic factors) [44]. Moreover, ATF4 protein has been linked to tumor microenvironment modulation, as it was reported to upregulate IL-6 and VEGF (potent angiogenic factors) in cancer cells [91].

As shown in Figure 10A the maximum increase of GRP78 protein in stressed cells occurred at longer exposure times (TUN 24h, -GLU 24h). During ER stress, GRP78 dissociates from the UPR-sensors, IRE1, PERK and ATF6, and bind to misfolded proteins to minimize aggregation processes; moreover, the activation of the PERK-UPR branch results in a rapid attenuation of mRNA translation in order to prevent the influx of newly synthesized polypeptides into the stressed ER [84]. Besides this general translation attenuation, specific proteins with uORFs such as ATF4, ATF5, CEBPA and CEBPB are selectively translated [49,92]. These transcription factors are responsible for the upregulation of some proteins involved in the reestablishment of ER homeostasis, such as GRP78, ERP1, and HMOX but also control the expression of pro-apoptotic factors like CHOP. CHOP leads the cell to cell cycle arrest and/or apoptosis [93] and is canonically viewed as the ultimate event of an intense and long activation of PERK-eIF2-ATF4 UPR axis. In our experiments, although increased levels of ATF4 were detected in tunicamycin-treated cells, concomitant upregulation of CHOP was not observed. Accordingly, Harding and co-workers have shown that in *Perk-/-* fibroblasts, forced ATF4 overexpression in absence of an additional stress stimulus was not sufficient for CHOP induction. Therefore, our results reinforce the idea that additional stress signals other than those transmitted by the PERK-eIF2α-ATF4 branch are required for CHOP induction in response to ER stress. Another possible explanation is the partial recovery observed in tunicamycin-treated cells, where ATF4 levels significantly decrease after 24h (Figure 10). This hypothesis is also supported by the results obtained in glucose deprived cells: given that glucose starvation lead to a sustained activation of the PERK-eIF2α-ATF4 this resulted in significant increase in CHOP expression (Figure 10).

Additionally, we analysed the effects of tunicamycin and glucose deprivation in the transcription of *CHOP*, *GRP78* and *ATF4* genes.

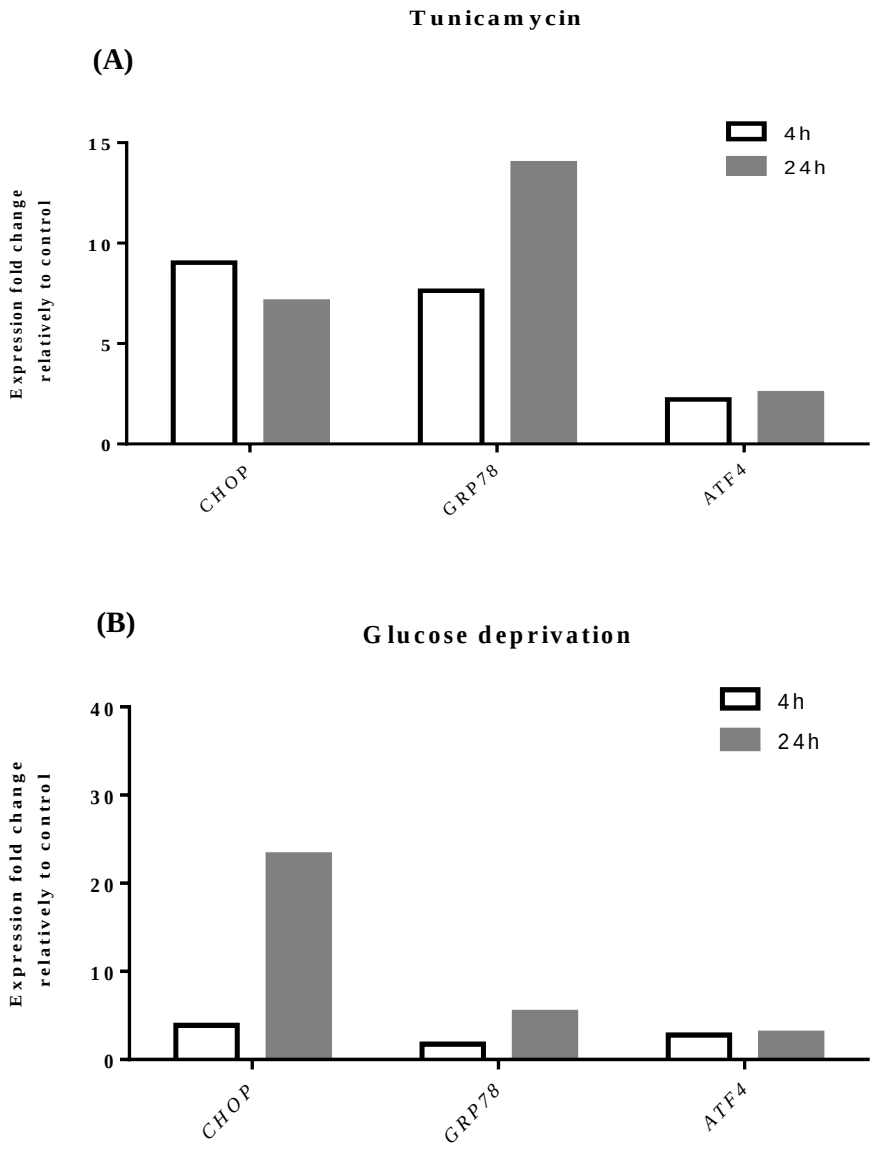


Figure 11 – Effect of tunicamycin (A) and glucose deprivation (B) on *CHOP*, *GRP78* and *ATF4* mRNA levels. T-47D cells were stimulated with tunicamycin (A) and glucose deprivation (B) for 4 or 24h. RNA was extracted and qRT-PCR performed to assess *CHOP*, *GRP78* and *ATF4* mRNA levels. The relative expression of the indicated genes was normalized using *TATA* as reference gene.

In accordance to the results obtained in protein analysis, qRT-PCR data revealed that transcription of *CHOP* and *GRP78* was effectively induced either by tunicamycin or glucose deprivation (Figure 11). Regarding *ATF4* gene we observed that the transcription is just slightly upregulated by both stimuli despite the high detected protein levels. This indicates that the observed increase of ATF4 protein results from a posttranscriptional event. Accordingly, it has been shown that during UPR activation the PERK-dependent phosphorylation of eIF2α causes a generalized attenuation of protein translation while

ATF4 translation is enhanced without changes in its mRNA transcription [94,95]. It is also worth highlighting that glucose deprivation stimulus requires a longer time of exposure to induce a stronger UPR activation on T-47D cells. Glucose deprivation is a milder stimulus comparing to tunicamycin that acts by directly inhibiting an essential process for the correct protein folding in the ER lumen. Thus its effective action probably requires longer incubation time in order to obtain more detectable changes in ER stress profile. Taken together, these data reveals that glucose deprivation effectively induces several arms of the UPR in T-47D cells and is therefore a good physiological stimulus to study the transmissible ER stress phenomena.

3. ER stress strongly induces interleukine-8 upregulation in breast cancer cells

The effect of ER stress on cancer cells cytokine profile was also assessed, in order to correlate with a possible signalling transmission to THP-1 cells in later experiments. To evaluate the expression of several cytokines under ER stress, T-47D cells were stimulated for 4 or 24h with each stimulus (tunicamycin or glucose deprivation), mRNA was extracted and *IL1β*, *IL8*, *CXCL14*, *VEGFA* and *VEGFB* transcription was analysed by qRT-PCR.

The ER stressors (tunicamycin and glucose deprivation) did not significantly affect the cytokine profile of T-47D cells, except for *IL8* expression (Figure 12). We observed a strong upregulation of *IL8* both for tunicamycin (23 fold) and for glucose deprivation (80 fold) treatment. Cancer cells have a typical inflammatory phenotype, characterized by the production and secretion of high levels of proinflammatory mediators as IL-6, PGE2, and chemokine as IL-8 and CXCL14, for example [96,97]. These mediators are responsible for the tumor growth, neovascularization and all the processes that support tumor survival and development. VEGF and IL-8 are potent angiogenic factors and are well characterized in human breast cancer [98,99]. Previous studies have shown that ER stress could be responsible for the upregulation of the pro-angiogenic factor IL-8 in human breast carcinoma [99], namely the axis PERK-eIF2α-ATF4 [100] . It is worth to note that IL-8 is strictly regulated by NF-κB cascade, which, in turn, has been associated with the activation of eIF2α, establishing that way a connection between UPR activation and NF-kB signalling pathways [101]. In fact ER stress-sensors and UPR-related proteins have been linked to the regulation of other inflammatory signalling cascades, namely: 1) the upregulation of IL-8

through activation of NF-κB mediated by CHOP (PERK-eIF2α-ATF4-CHOP) [102]; 2) the upregulation of TNF-α, IL-6, IL-8 and MCP-1 through activation of JNK/p38 MAPK pathway by IRE1 (IRE1-TRAF2-ASK1) [103]; 3) the upregulation of TNF-α, TGF-β, IL-2, IL-6 and IL-8 through activation of NF-kB by IRE1(IRE1-TRAF2-IKK)[104].

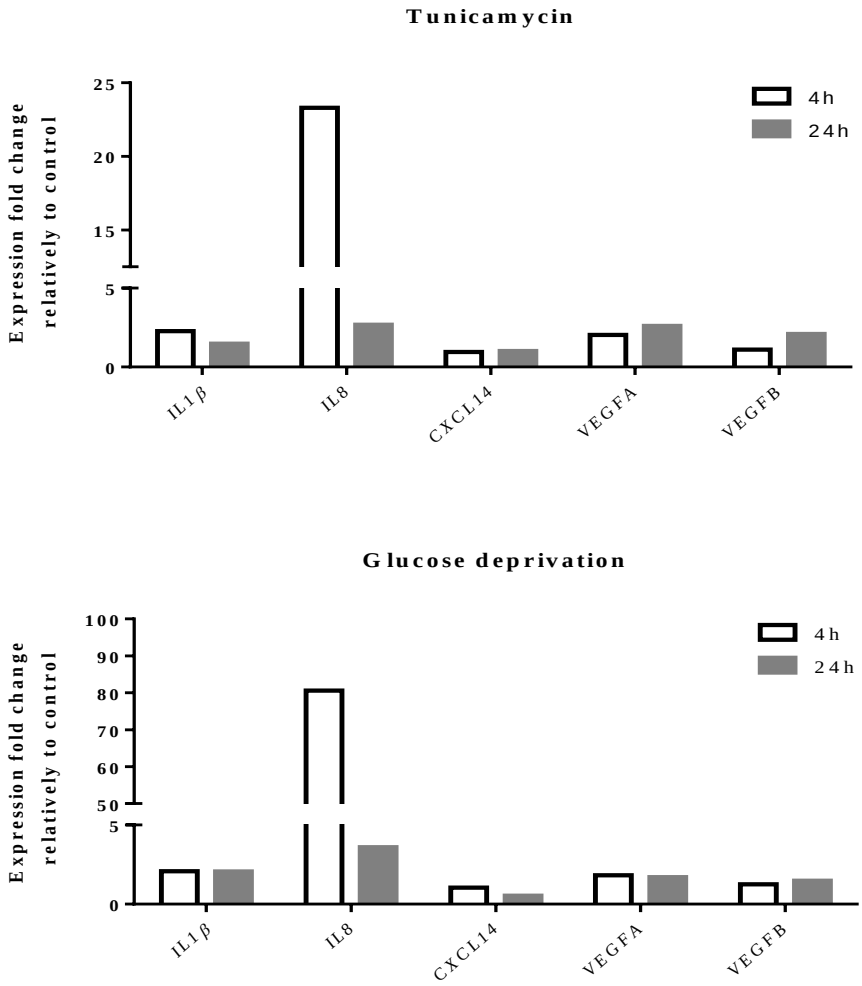


Figure 12 – Effect of tunicamycin and glucose deprivation in mRNA levels of *IL1β, IL8, CXCL14, VEGFA* and *VEGFB*. T-47D cells were stimulated with tunicamycin (A) and glucose deprivation (B) for 4 (white bars) or 24 (grey bars) hours. *IL1β*, *IL8*, *CXCL14*, *VEGFA* and *VEGFB* mRNA levels were normalised against control (cells unstimulated).

4. Conditioned medium from glucose deprived T-47D cells does not induce ER stress in THP-1 cells

Our main goal in this work was to determine how ER stressed breast tumor cells could transmit ER stress to other cells present in the tumor microenvironment such as macrophages and dendritic cells, in order to modulate their immunostimulatory abilities. The concept of "Transmissible ER Stress" (TERS) was established by Mahadevan and collaborators when they observed that conditioned medium (CM) from ER stressed cancer cells induced UPR activation in myeloid cells, namely increased expression of GRP78 [77]. They also observed that myeloid cells cultivated with CM from ER stressed cancer cells underwent through an immunosuppressive modulation and also shifted their inflammatory profile, namely towards a proinflammatory phenotype. However, the mechanisms that outline this transmission were not clarified in none of the referred works.

As a first approach we recovered the CM from each T-47D growth condition, centrifuged it to discard dead cells and cellular remains, and incubated THP-1 cells with respective CM for 24 hours, as outlined in Figure 13.

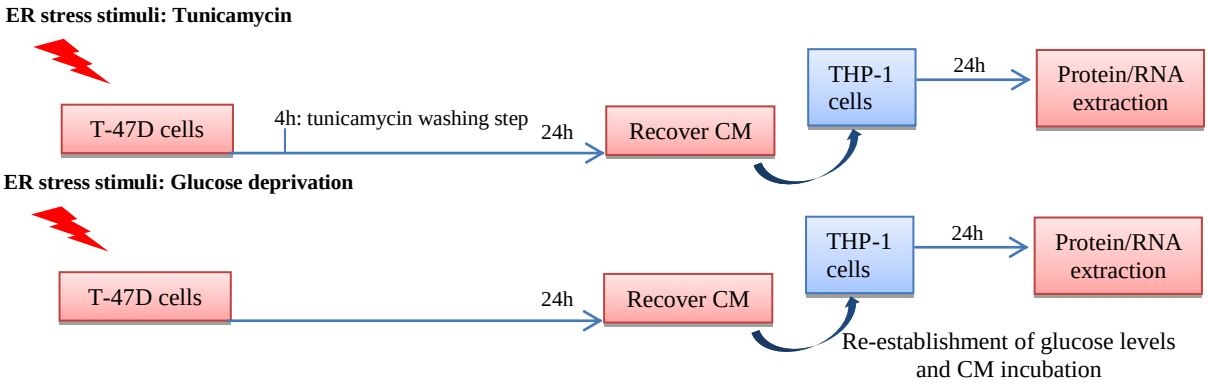
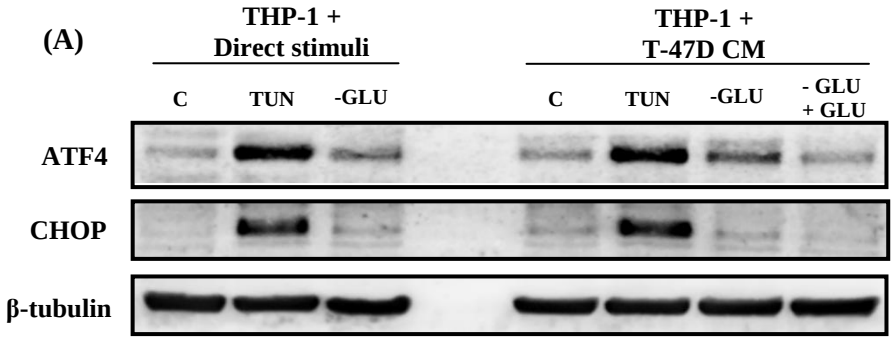


Figure 13 – Schematic representation of the methodology applied to assess transmission of ER stress by T-47D-derived CM. ER stress was induced in T-47D cells by tunicamycin- or glucose deprivation-CM. After 4h of incubation with tunicamycin, T-47D cells were washed and incubated with fresh medium for more 24h.



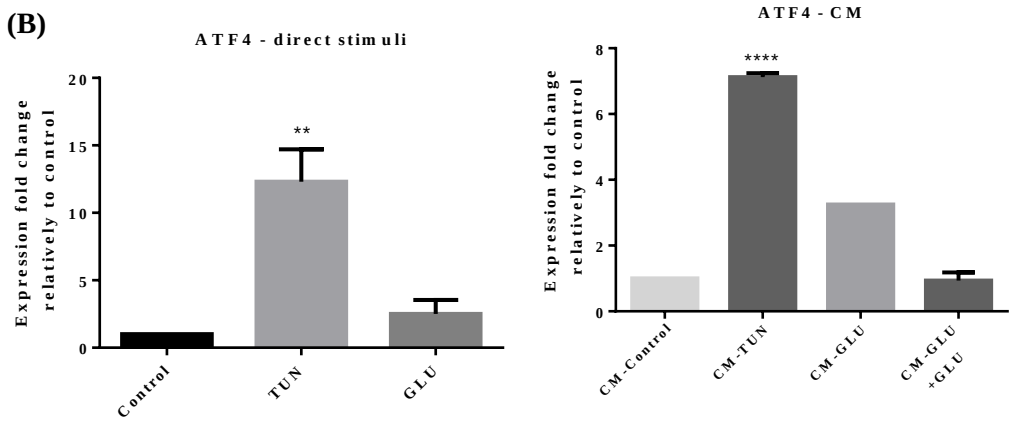


Figure 14 - Effects of glucose deprivation- and tunicamycin-CM on the levels of ATF4 protein in THP-1 cells. (A) THP-1 cells were incubated for 24h with glucose deprivation-CM (with reposition of glucose, CM-GLU+GLU or without, CM-GLU) and tunicamycin-CM (CM-TUN). Direct stimulation with ER stressors was also performed to control THP-1 responsiveness. **(B)** The results represent at least 3 independent experiments (except for CM-TUN that represents only 1) and were expressed as fold changes relatively to control (cells without any treatment). *p<0.1; **p<0.01; ****p<0.0001

As shown in Figure 14, conditioned medium from tunicamycin-treated T-47D cells effectively induced ER stress in THP-1 recipient cells. This result was in agreement with the previous study of Mahadevan and collaborators, and suggests that ER stress may be transmitted between cells through some soluble factors released into the microenvironment. In the referred study, several cancer cell lines were treated with thapsigargin (a pharmacological ER stress inducer) and their conditioned medium was used to culture myeloid cells [42]. The activation of the UPR in recipient cells was demonstrated by the increased transcription of GRP78, CHOP, and XBP1-s. In the same study, the authors also performed a punctual experiment where they induced ER stress in cancer cells by glucose deprivation. They used this glucose free conditioned medium to culture myeloid cells and observed that they also developed characteristics of ER stress. As the mRNA levels of ER stress markers such as CHOP, GRP78 and XBP1-s were slightly higher in cells that received the glucose free conditioned medium compared to cells directly cultured in fresh medium without glucose, they concluded that ER stress was also transmitted in these conditions (11). However, in our perspective, a key experimental condition was lacking in these experiments: myeloid cells cultured in conditioned medium from glucose starved cancer cells but where the levels of glucose were re-established prior treating the myeloid cells (glucose 25 mM). To clarify this hypothesis we performed an

assay to compare both the procedures: an experiment in which we restored the glucose levels to 25 mM (CM-GLU+GLU), and a second one, reproducing Mahadevan work conditions, in which THP-1 cells were incubated with glucose deprived conditioned medium without reestablishment of glucose levels (CM-GLU). As shown in Figure 14, THP-1 cells cultured in glucose free T-47D-conditioned medium (CM-GLU) present increased levels of ATF4, and these levels are slightly higher than in cells directly deprived. This slight overexpression may be related to the presence of soluble factors, such as cytokines, released by ER stressed T-47D cells and present on CM-GLU. However, if glucose is restored (CM-GLU+GLU) this effect is almost completely abolished. These results clearly evidence that in cells cultured in the glucose free conditioned medium the development of ER stress is mainly a direct consequence of the lack of glucose rather than a transmission of UPR-involved signalling factors.

Of note is the fact that even though glucose deprivation (direct stimulus) also caused ER stress in THP-1 cells, the levels of UPR effectors such as ATF4 and CHOP (Figure 14) were substantially lower than the ones observed in T-47D cells (Figure 10). Despite the high requirements of cancer cells for glucose-dependent metabolism [8], THP-1 cells are characterized with a lower proliferative rate and glycolytic activity, which results in a lower dependence of glucose. These metabolic characteristics may explain the different response magnitude towards glucose levels observed for T-47D and THP-1 cells.

From our results an intriguing question arises: if tunicamycin and glucose deprivation are equally effective in inducing ER stress in T-47D cells why ER stress transmission was only observed in tunicamycin-CM recipient cells? The most plausible explanation may be the co-transport of the drug by the conditioned medium either as free tunicamycin or vesicle-associated tunicamycin. Mahadevan and collaborators have excluded the hypothesis of thapsigargin (also a pharmacological ER stress inducer) co-transport through CM once mass spectrometry analysis did not shown any peak relative to this compound; however, they analysed an extremely low CM volume (300 µL), leading to a possible misconception of the presence of the drug in CM, which concentration could be under the detection limit of the technique.

5. Characterization of T-47D cell-derived exosomes

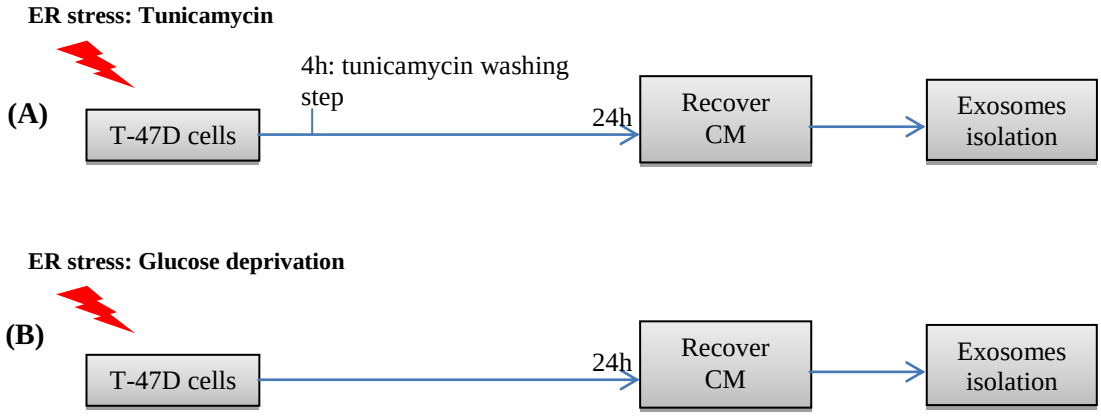


Figure 15 – Schematic representation of the methodology applied to isolate T-47D ER stress exosomes. ER stress was induced in T-47D cells by tunicamycin (A) and glucose deprivation (B). After 24h, exosomes were isolated and purified from the CM of T-47D ER stress cells with Exo-spin™ kit.

To clarify the possible mechanism of ER stress transmission observed in tunicamycin conditioned medium we decided to isolate the stressed T-47D cell-derived exosomes (Figure 15). Exosome isolation has become a very useful procedure in research, due to interesting findings regarding the role of these small vesicles in cell-cell communication. The most ancient method to isolate exosomes (and the most used) is the ultracentrifugation process; however it requires an ultracentrifuge machine, is extremely time consuming and usually results in very low yields of exosomes [105]. We opted to use a recent developed exosome isolation kit from Cell Guidance Systems in order to achieve higher yields of purified exosomes. The classical characterization of exosomes is based on the size (around 50-200 nm of diameter), the morphology (round shaped membrane vesicles (reviewed in [79])) and the presence of some membrane surface proteins, such as CD81 and CD63 [106]. Thus we performed Western blot technique to detect these two markers (Figure 16).

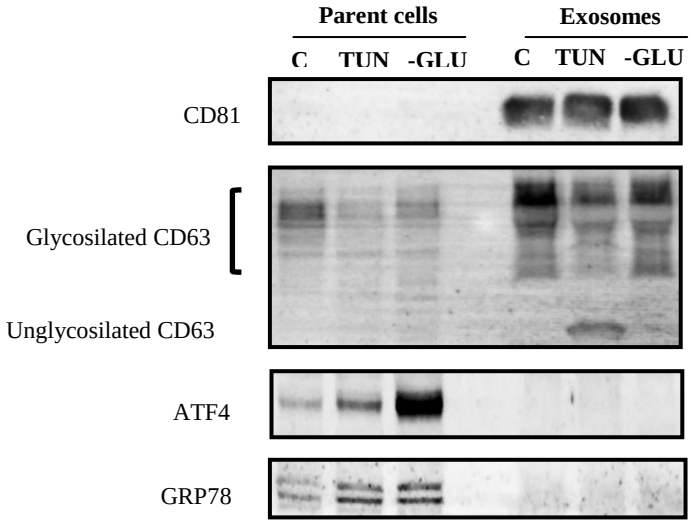


Figure 16 - Characterization of exosome by Western blot. Samples were tested for typical exosomal markers, CD81 and CD63, and also for UPR markers, as ATF4 and GRP78. Parent cells were stimulated with tunicamycin and glucose deprivation medium and the released exosomes were isolated from conditioned medium after 24h using Exo-spin™ kit. Parent cells Western blots were performed to comparision with exosomal fraction.

As presented in Figure 16, protein extracts from exosomes (from control-, tunicamycin- and glucose deprivation-conditioned medium) shown to be enriched in CD81 and CD63 tetraspanins compared to the respective parent cells. Additionally, we observed that in exosomes from tunicamycin-treated cells CD63 is present in both unglycosylated (25kDa) and glycosylated (36 to 60kDa) forms [107]. This particular result indicates that tunicamycin is inducing ER stress in parent cells, by effectively inhibiting the N-glycosylation of proteins, leading to the accumulation of misfolded proteins in the ER lumen. Moreover, this finding also demonstrates that exosomes from ER stressed cells carry protein factors that reflect the condition of the cells that originate them.

Exosomes are only possible to directly visualize through electron microscopy due to their small size. Thus, we performed an observation of the ultrastructure of putative exosomes using Transmission Electron Microscopy (TEM). In Figure 17 we can observe a representative TEM image of the isolated nano vesicles.

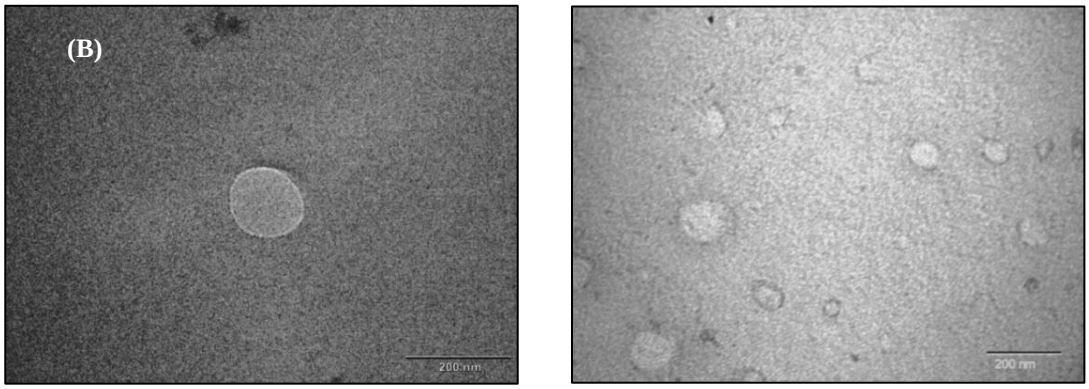


Figure 17 – Representative Transmission Electron Microscopy image of T-47D-derived exosomes ultrastructure. Exosomes were isolated from T-47D cells grown in RPMI medium by Exo-spin™ kit. Morphologically the exosome-like vesicles are within the typical size range ((A) ~160 nm, (B) 60-140 nm), spherical and limited by a well-defined lipid layer. (B) Observation of cup-shaped morphology is also typical in exosome TEM images.

The majority of the visualized vesicles (Figure 17) were within 40-200 nm size range, indicative of exosomes [106,108], suggesting that our exosomes were successfully purified.

In order to understand the factors beneath exosomal action in THP-1 cells, we performed an electrophoretic separation of exosomal and parent cells proteins (Figure 18).

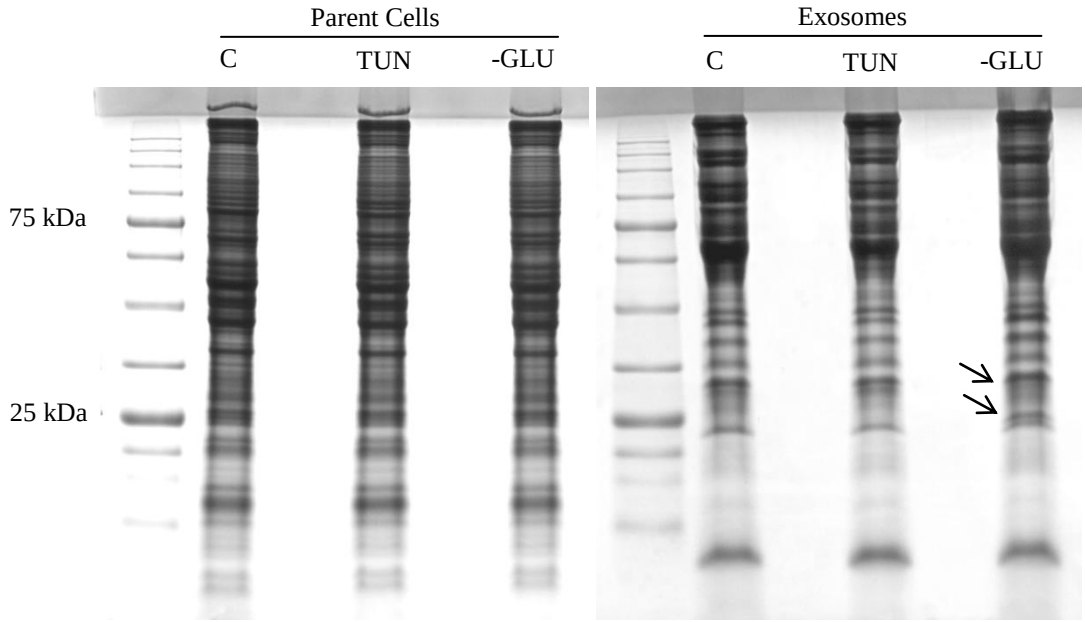


Figure 18 – SDS-PAGE gel electrophoresis of the proteins from T-47D total cell lysates and T-47D cell-derived exosomes from different treatments (tunicamycin and glucose deprivation). T-47D cells were

incubated with tunicamycin and/or glucose deprived medium (tunicamycin was washed after 4h of incubation). After 24h, exosomes were isolated and purified as well as total cell lysates. 50 µg of protein were then run on SDS-PAGE gel.

Despite the protein identification is still in progress, we expect to see relevant differences between control and stressed exosomes. It is clear that bands pattern namely for glucose deprivation exosomes, is slightly different from both tunicamycin and control exosomes: we can observe the appearance of a new band at approximately 25kDa as well as the absence of a 37 kDa band. These inferences are only plausible with the support of protein identification and quantification, which is already in progress by MALDI analysis.

6. Exosomes from glucose deprived T-47D cells do not induce ER stress in THP-1 cells

The role of tumor-derived exosomes has been increasingly studied and discussed. Earlier studies demonstrated that tumor-derived exosomes essentially carry tumor antigens, which result in enhanced activation of immune responses; however, recently the link between cancer cells-derived exosomes and tumor growth and development has been focused in possible immunosuppressive abilities. To identify the effects of T-47D-derived exosomes on the modulation of dendritic cells, and the possible transmission of ER stressors we first evaluated the UPR activation in recipient THP-1 cells. To that end, we isolated the exosomes originated from tunicamycin or glucose deprived T-47D cells and cultured monocytes with purified exosomes for 24h (Figure 19).

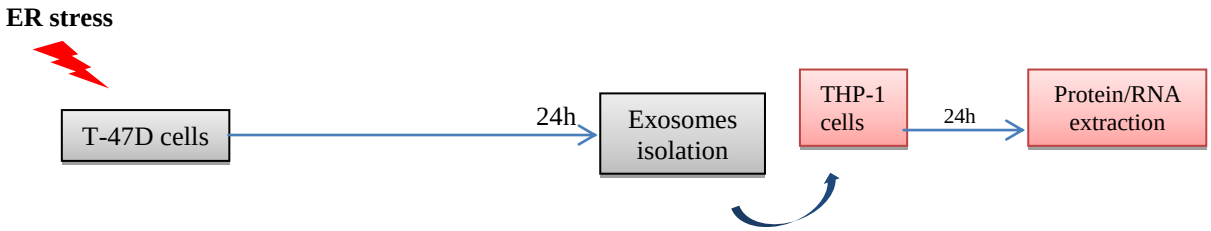


Figure 19 – Schematic representation of the methodology applied to assess THP-1 induced ER stress by T-47D-derived exosomes. ER stress was induced in T-47D cells by tunicamycin and glucose deprivation. After 24h of incubation, exosomes were isolated and purified from ER stress CM and THP-1 were incubated for 24h with the respective eluate-containing exosomes.

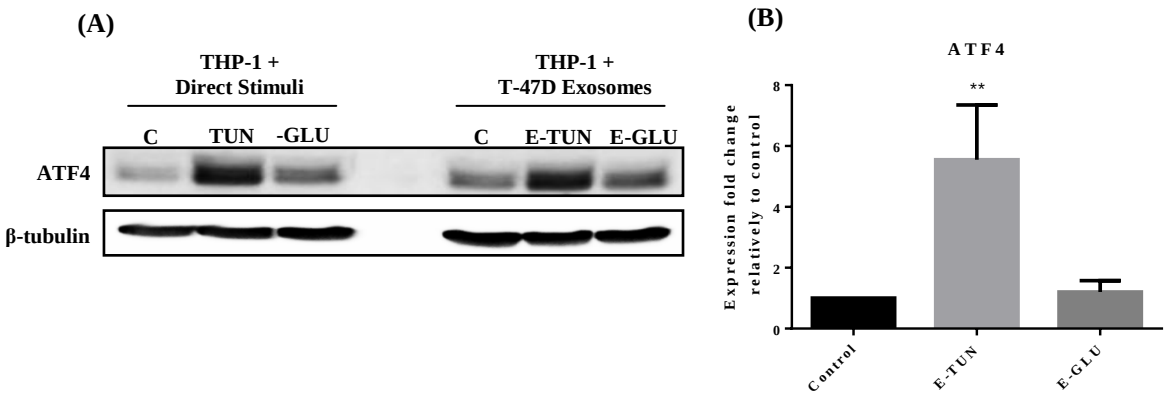


Figure 20 - Effect of exosomes from tunicamycin- and glucose deprived-T-47D cells on UPR activation in THP-1 cells. (A) THP-1 cells were incubated with exosomes from the respective T-47D-stimulated ER stressor (tunicamycin (E-TUN) or glucose deprivation medium (E-GLU)) for 24h as schematized in Figure 19. Western blotting was performed to assess ATF4 protein expression. For reference THP-1 cells were also directly stimulated with each stimulus (TUN and –GLU) for 24h, in the same conditions. **(B)** The results represent 4 independent experiments and were normalized against control (cells untreated). **$p<0.01$

The protein analysis showed that exosomes from tunicamycin treated cells induced a significant increase of ATF4 expression (5.548 ± 0.9027) in THP-1 recipient cells (Figure 20). This result may explain the observed transmission of ER stress when cells were exposed to tunicamycin conditioned medium and strongly advises against the TERS phenomena. Moreover exosomes from glucose deprived medium (E-GLU) did not induce relevant differences relatively to the control. Thus, it seems that the transmission of ER stress is only noticeable when pharmacological inducers such as tunicamycin or thapsigargin are used. This results not from the transmission of some cell stressor factors but from the transmission via exosomes of the drugs itself. To further investigate if ER stress was induced via exosomal carrying, we analysed the ATF4 levels on THP-1 cells incubated with tunicamycin stressed T-47D cells secretome: complete conditioned medium, conditioned medium depleted from exosomes and isolated T-47D cells-derived exosomes (Figure 21). As expected, we observed that cells exposed to exosomes-depleted medium showed a dramatically decrease of ATF4 levels (Figure 22). Thus we can conclude that the factor responsible for the induction of ER stress in THP1 cells is within the exosomes.

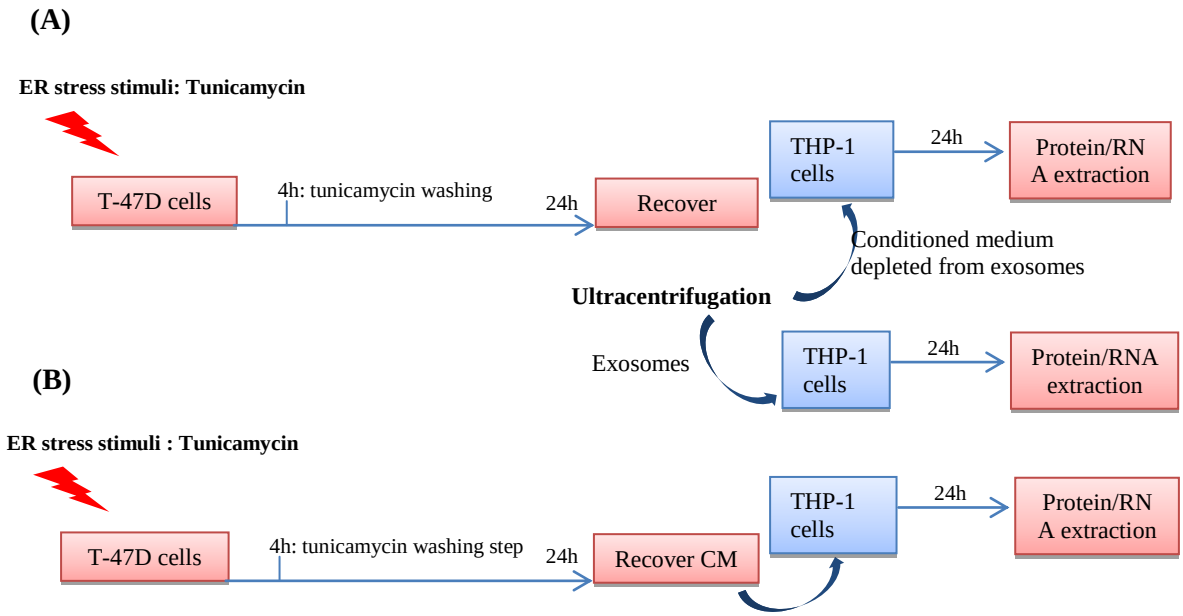


Figure 21 - Schematic representation of the methodology applied to assess THP-1 induced ER stress by T-47D-derived differentially secretome. ER stress was induced in T-47D cells by incubation for 4h. Then T-47D cells were washed with fresh medium. After 24h of incubation, conditioned medium was recovered and THP-1 were incubated for 24h with exosomes (A), conditioned medium depleted from exosomes (A) and complete conditioned medium (B). Exosomes were isolated from conditioned medium by ultracentrifugation.

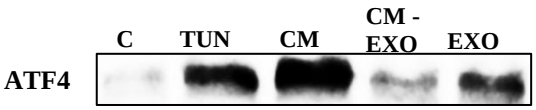


Figure 22 - Effect of differential secretome from tunicamycin-treated T-47D cells on UPR activation in THP-1 cells. THP-1 cells were incubated with conditioned medium (CM), conditioned medium depleted from exosomes (CM-EXO) and exosomes (EXO) from T-47D cells stimulated tunicamycin with for 24h as schematized in Figure 21. Western blotting was performed to assess ATF4 protein expression. For reference THP-1 cells were also stimulated with tunicamycin (TUN) for 24h, in the same conditions.

UPR-related gene expression was also investigated by evaluating *ATF4*, *CHOP* and *GRP78* mRNA levels through qRT-PCR. In accordance to the protein data, tunicamycin-related exosomes induced the upregulation of *ATF4*, *GRP78* and *CHOP* mRNA on THP-1 cells (E-TUN) (Figure 23). Again, this effective induction of ER stress through exosomes from T-47D cells treated with tunicamycin corroborates our previously formulated hypothesis, that tunicamycin could possibly be transported inside exosomes. On the other hand, the transcription of studied genes did not increased significantly in cells treated with glucose deprivation-related exosomes (E-GLU). Thus, we can conclude that T-47D cells

are not able to transmit ER stress through exosomal carrying at least for physiological inducers.

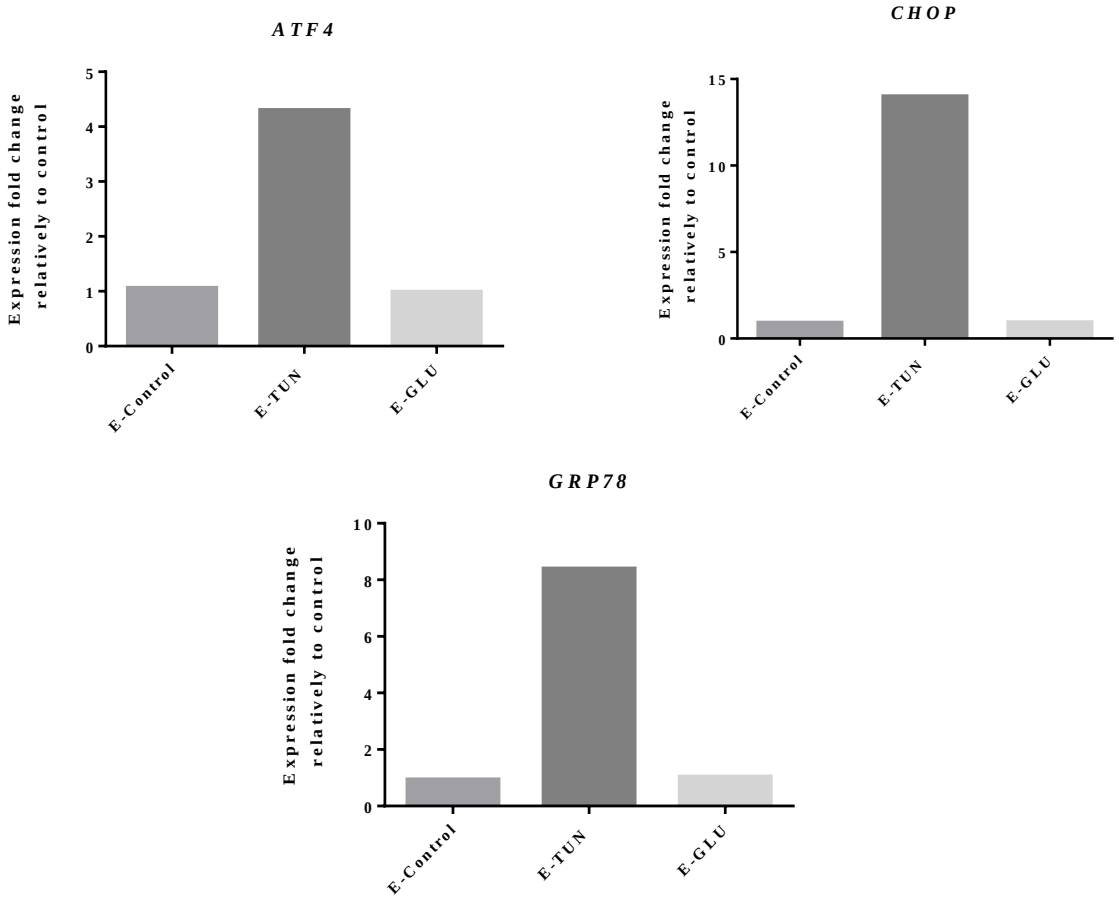


Figure 23 - Effect of tunicamycin- and glucose deprivation-exosomes on *ATF4*, *CHOP* and *GRP78* mRNA levels in THP-1 cells. THP-1 cells were incubated with exosomes from the respective T-47D-stimulated ER stressor (tunicamycin (E-TUN) or glucose deprivation medium (E-GLU)) for 24h as schematized in Figure 19. qRT-PCR was performed to assess *ATF4*, *CHOP* and *GRP78* mRNA expression.

7. T-47D cell-derived exosomes modulate THP-1 cells cytokine production and maturation status

As previously suggested, ER stressed T-47D-derived exosomes could be playing an important role in the immunosuppression of dendritic cells. In order to study the modulation of DCs phenotype, we evaluated the mRNA levels of several inflammatory cytokines and chemokines (*IL1β*, *IL6*, *IL8*) and also a maturation marker (*CD86*) by qRT-PCR. Additionally, in a parallel experiment, THP-1 cells were pre-treated with LPS (a

potent activator of immune system) in order to increase the magnitude of the observed differences, and also to understand the effect of exosomes on cell response.

DCs treated with E-GLU did not show significant variation of cytokine expression (Figure 24), except for *IL1β* mRNA which was slightly down-regulated (9.6 fold). Despite we acknowledge the unresponsiveness of THP-1 cells to glucose deprivation (direct stimulation and/or through exosome incubation), we observed a different expression profile when THP-1 cells were pre-treated with LPS, namely downregulation of *CD86* (1.5 fold) and *IL6* (15 fold) and upregulation of *IL1β* (1.5 fold) and *IL8* (1.8 fold). This suggests that E-GLU induces a slight modulation in THP-1 cells, which is only detectable when monocytes are activated. The decreased expression of *CD86* is characteristic of tolerogenic dendritic cells, which are incapable to polarize T cells through a cytotoxic phenotype. High levels of tolerogenic dendritic cells lead to an accumulation of $CD4^{+}CD25^{+}$FOXP3 regulatory T cells [34,109]. This immunoescape mechanism is a consequence of tumor-derived exosome modulation, as revealed by Yu and co-workers. They observed that tumor-derived exosome attenuated DC differentiation and $CD11^{+}$c DCs were characterized by low levels of CD86 protein [109]. As UPR activation was not detected in THP-1 incubated with E-GLU (Figure 23) we can propose that THP-1 modulation is ER stress-independent and must result from the transport of cancer-associated factors, as cytokines and/or microRNA. In turn, upregulation of *IL8* is very prominently on THP-1 cells incubated with E-Control and E-GLU. IL-8 is a hallmark chemokine of tumor pro-inflammatory mediators, known by its potent angiogenic character [99]. Our analysis of inflammatory profile in stressed and unstressed T-47D cells (Figure 12) reported an upregulation of *IL8* mRNA in both tunicamycin-treated and glucose deprived cells. In conformity with our results, Baj-Krzyworzeka and collaborators showed high levels of *IL8* mRNA within tumor-derived microvesicles, being this related to the observed modulation of monocyte cytokine production [110]. This parallelism between *IL8* mRNA levels in T-47D (Figure 12) and THP-1 cells (Figure 24) indicates that tumor-released exosomes can be driving THP-1 cells towards a supportive role on the angiogenic demand of T-47D cells.

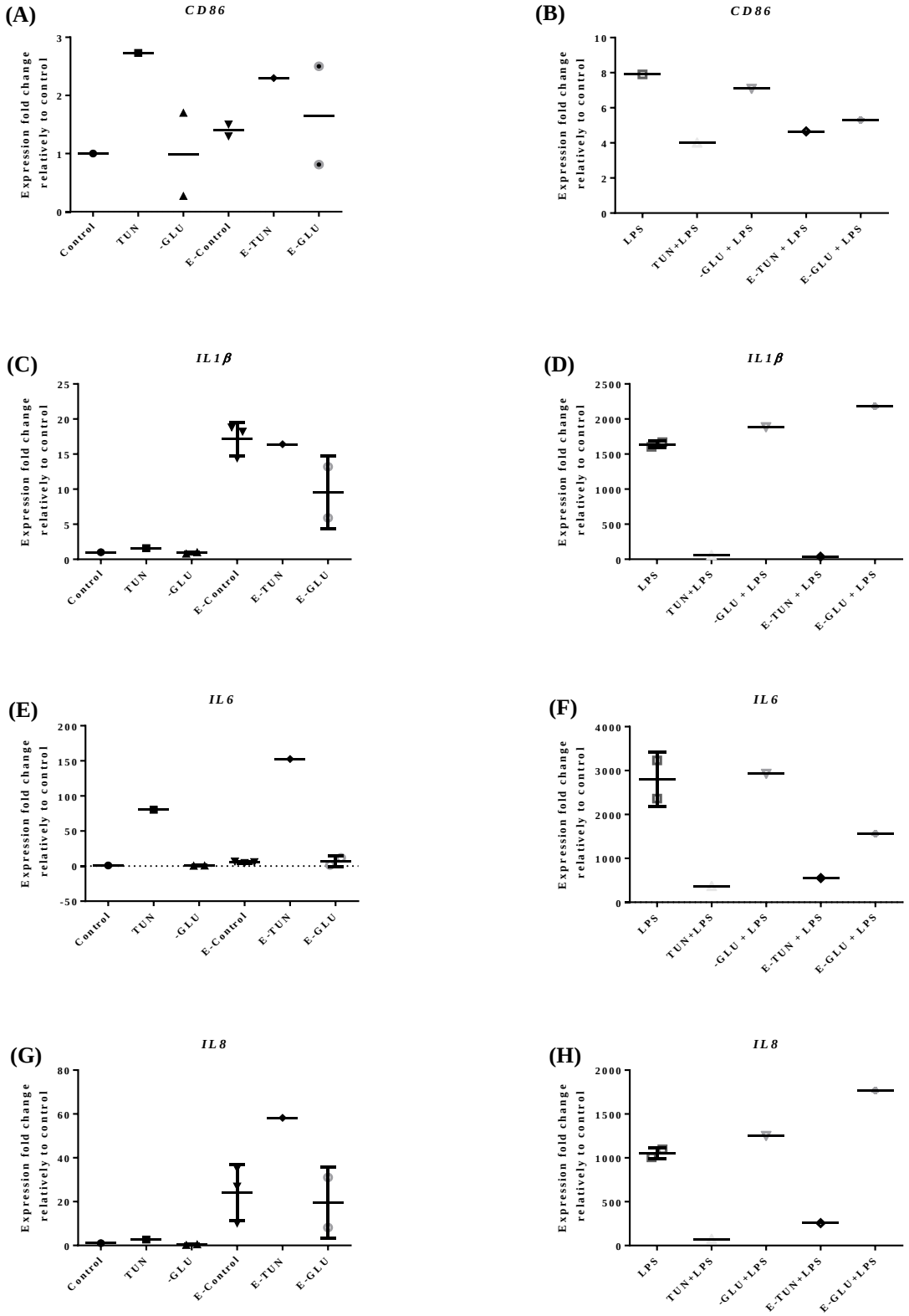


Figure 24 – Effect of T-47D cells-derived exosomes in *CD86, IL1β, IL6 and IL8* mRNA levels of THP-1 recipient cells. THP-1 cells were incubated with exosomes derived from ER stressed T-47D cells or unstressed cells for 24h (A, C, E, G). In parallel, THP-1 cells were pre-stimulated with LPS for 4h before

incubation with exosomes (B, D, F, H). Therefore, qRT-PCR was performed for detection of *CD86* (A,B), *IL6* (C,D), *IL1β* (E,F) and *IL8* (G,H) mRNA cytokines.

As expected, E-TUN induced a generalized upregulation of proinflammatory mediators, such as *IL6* (80 fold) and *IL8* (2.7 fold) and maturation marker *CD86* (2.7 fold). Basal levels of cytokines (*IL6*, *IL*8 and *IL10*) mRNA were already reported to be significantly higher in ER stressed cells [111]. It is important to note the similarity of cytokine regulation pattern observed for THP-1 incubated with E-TUN and direct stimulation with tunicamycin, except for the upregulation of *IL8* in E-TUN; once again these results support our hypothesis that tunicamycin is transported through exosomal-carrying and directly stimulates THP-1 cells. Thus, it is plausible to assume that cytokine upregulation in THP-1 cells is a direct consequence of tunicamycin action in monocytes, and consequently, an ER stress-dependent modulation. Nonetheless after pre-treatment with LPS, E-TUN downregulates cytokine and *CD86* mRNA expression in THP-1 cells, indicating an attenuation of the inflammatory phenotype. ER stress is highly associated with inflammatory mechanisms, namely the activation of NF-κB cascade [70]. As recently reported by Zhang and collaborators, ATF4 plays a crucial role on TLR4 signalling, resulting on enhanced cytokine production [112]; however, the effect of tunicamycin, a pharmacological ATF4 inducer, results on inhibition of NF-κB cascade on THP-1 cells treated with LPS. This inhibition was explained by the deleterious effect of tunicamycin in TLR4 signalling, attenuating the proinflammatory cytokines expression [113].

Overall our data indicates that TERS phenomena seems to be a misconception resultant from the transport of pharmacological inducers (tunicamycin in this particular case) within the exosomes and the direct UPR activation in monocytes. We also acknowledged that, despite exosomes from glucose deprived cells are not able to induce ER stress on target cells, they efficiently modulate the inflammatory profile and maturation status of THP-1 cells.

Chapter II - Lactic acidosis mitigates glucose deprivation-ER stress in T-47D cells

In our first approach to induce ER stress in T-47D cells (Figure 9), we tested different conditions normally present in solid tumor microenvironment such as glucose deprivation and lactic acidosis. Surprisingly, we observed that cells simultaneously cultured in glucose deprived medium and lactic acidosis (pH 6.6, 25mM lactic acid), presented significantly low levels of ER stress than solely glucose deprived cells. After exploring some literature we decided to, parallel to the main aim of this project, perform some experiments to clarify a possible mitigation of glucose deprivation-ER stress, driven by lactate. To further study this possible mechanism, we compared the effect of lactate, glucose deprivation + lactate and glucose deprivation + hydrochloride acid (HCl), an inorganic acid.

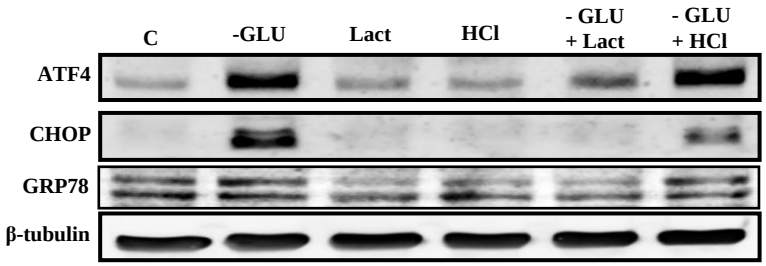


Figure 26 – Effect of glucose deprivation, lactate, hydrochloride acid and conjugation of each acid with glucose deprivation on ATF4, CHOP and GRP78 protein levels of T-47D cells. T-47D cells were stimulated for 24h with glucose deprived medium (-GLU), RPMI 10% FBS supplemented with 25mM lactate pH6.5 (Lact), hydrochloride acid pH6.5 (HCl), glucose deprived medium + 25mM lactate pH6.5 (-GLU+Lact) and glucose deprived medium + hydrochloride acid pH6.5 (-GLU+HCl). Then, total protein extracts were prepared and ER stress evaluated through analysis of ATF4, CHOP and GRP78 protein levels by Western blotting.

Both lactate and HCl stimulation did not induce relevant levels of ER stress in cancer cells, as mentioned and discussed earlier. On the contrary, glucose deprivation efficiently induces ER stress, reflected by the overexpression of ATF4, GRP78 and CHOP; however, we observed a diminished expression of the aforementioned proteins in glucose deprivation + lactate cultured cells (-GLU+Lact) (Figure 26). We also analysed the effect of lactate in glucose deprived cells mRNA levels which were congruently with the protein data (Figure 27).

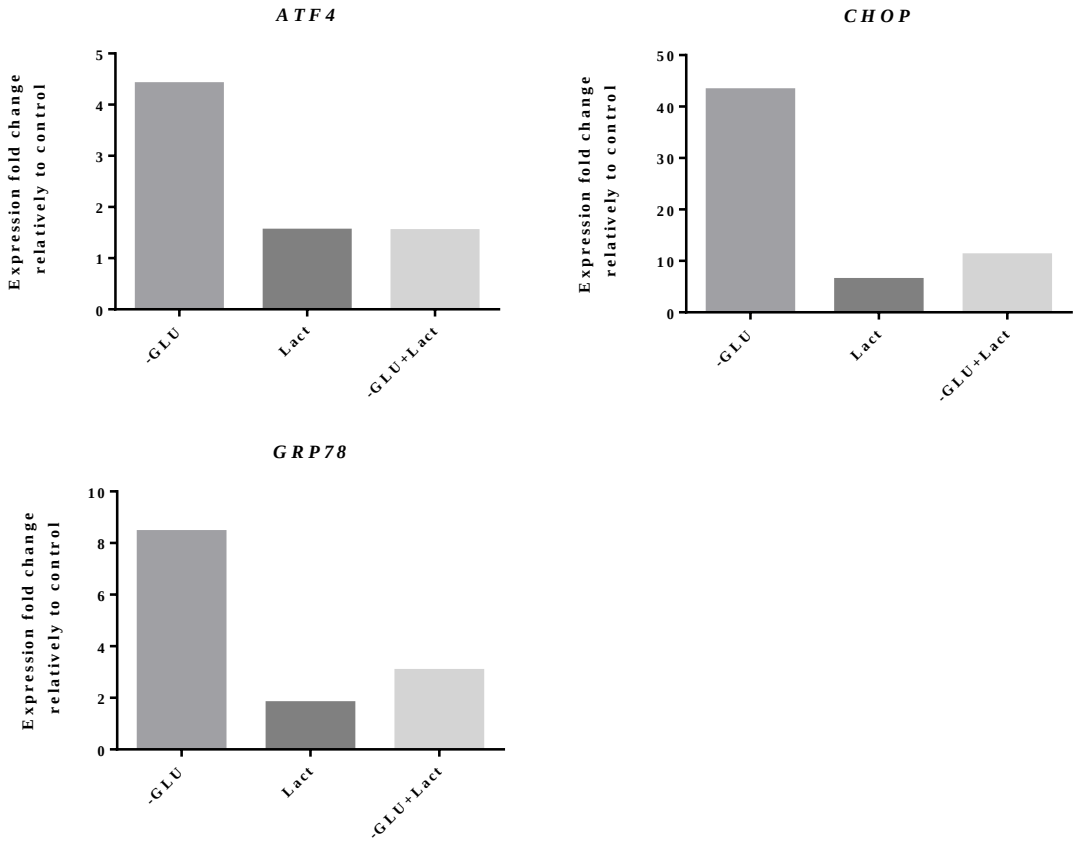


Figure 27 – Effect of glucose deprivation, lactate and conjugation of lactate with glucose deprivation on levels of *ATF4, CHOP* and *GRP78* mRNA. T-47D cells were stimulated for 24h with glucose deprived medium (-GLU), lactate (Lact) pH6.5 and glucose deprived medium + lactate pH6.5 (-GLU+Lact). RNA was extracted and qRT-PCR performed to assess *ATF4, GRP78* and *CHOP* mRNA levels. The relative expression of the indicated genes was normalized using *TATA* as reference gene

Accordingly to our hypothesis, mitigation of glucose deprivation-induced ER stress is not dependent of pH *per se*, as the acidification of glucose deprived medium with an inorganic acid (-GLU+HCL) did not revert the ER stress caused by the lack of glucose. We hypothesise that this mitigation could be consequence of a shift on cells metabolism, as lactate could be used as energy source in conditions where supply of glucose is restricted starved. Lactate is an organic acid and the principal product of anaerobic respiration in muscle cells under exercise endurance, low pO_2 and also glycolytic cancer cells [7]. The energy metabolism in tumor cells is peculiar and a subject of several studies, some of them quite controversial [114–118]. The traditional approach of cancer metabolism is based on the dysfunction of mitochondrial respiration, even in normoxic environment - the Warburg effect. To cross this impairment, cell forwards the glycolytic products (pyruvate) to the

action of lactate dehydrogenase (LDH), allowing cell to produce energy from glucose and resulting in the production of ATP and lactate, as final products [7,119]. Cancer cells have an extremely high proliferative rate and therefore an increased consumption of glucose. This demand is supported by the increased expression of glucose transporters (GLUT1) and also glycolytic and lactate dehydrogenase enzymes. Additionally, the resultant lactate molecules are transported to extracellular space through monocarboxylate transporters (MCT4) [120]. Despite this typical and well-known metabolic characterization, recent studies have suggested a new mechanism by which glucose metabolism is driven within the tumor microenvironment, the reverse Warburg effect [121]. This theory is based on the induction of Warburg effect in stromal cells of the tumor, by epithelial cancer cells. Cancer cells release high amounts of ROS that have an impact on stromal cells promoting oxidative stress and impaired mitochondrial respiration [122]. Aerobic glycolysis is favoured in stromal non-cancer cells and the final product, Lactate, is transported to the extracellular space. Then, cancer cells uptake these lactate molecules and use it as the preferred energy source, favouring the tricarboxylic acid (TCA) pathway [9,123]. Indeed, our results suggested that T-47D cells are not starved; we performed an immunofluorescence assay and did not detect apoptotic or necrotic cells in the GLU+Lact treatment (data not shown). Moreover, the lactate-dependent mitigation of ER stress in glucose deprived cells lead us to postulate that lactate could be re-directed to gluconeogenesis pathway in order to support cell grow and normal protein glycosylation. Recently two interesting reports have shown that mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M), a gluconeogenesis key enzyme present mainly in hepatocytes, is overexpressed in cancer cells [124,125]. Leithner and collaborators reported an enhanced expression and activity of PEPCK-M in lung cancer cells under low glucose conditions. Moreover they also labelled lactate and observed an accumulation of phosphoenolpyruvate, a precursor of gluconeogenesis, concluding that lactate may be used as gluconeogenic precursor in low glucose environment [124]. The mechanisms responsible for the PEPCK-M overexpression in those conditions are still unclear; however Mendez-Lucas and co-workers proposed that activation of GCN2-eIF2α-ATF4 and PERK-eIF2α-ATF4 (by amino acid depletion or UPR activation, respectively) have a crucial role in the signalling PEPCK-M expression [125].

With this parallel experiment we aimed to assess a potential mechanism and interaction driven by tumor cells in the scope of energy metabolism. As discussed before, we assumed that glucose deprivation would induce ER stress in cancer cells due to the impossibility to forward proteins through N-glycosylation. Therefore, the low ER stress levels observed in glucose deprived cells supplemented with lactate suggest that lactate is being driven through gluconeogenesis pathway, which increase the available glucose in the cell. Moreover, as T-47D cells continued to grow, it is plausible to hypothesize that lactate is also being used to ATP production through *tricarboxylic acid cycle (TCA) – oxidative phosphorylation* (OXPHOS) respiration. These two active pathways (gluconeogenesis and TCA-OXPHOS) are expected to be highly regulated in cells with high metabolic demands and it would be interesting to elucidate in future works the role of lactate in tumor survival.

Concluding remarks

With the present work we can conclude some important aspects of tumor microenvironment:

- High levels of tunicamycin induce ER stress in T-47D cells;
- Tumor microenvironment factors (as glucose deprivation, acidosis and hypoxia) induce ER stress in T-47D cancer cells;
- ER stress strongly induces upregulation of the angiogenic factor *IL8* in T-47D cells*;*
- TERS phenomena is not observed under physiological conditions;
- Tunicamycin is probably carried on T-47D cell-derived exosomes which directly induces ER stress on THP-1 cells.
- Exosomes from glucose-deprived T-47D cells modulate THP-1 cells towards a particular proinflammatory profile and decreased maturation status.

Additionally, parallel work has revealed some new aspects about tumor metabolism, namely that:

- Lactate mitigates ER stress induced by glucose deprivation.

Outlook

It is important to clarify how tunicamycin-induced ER stress is transmitted to THP-1 cells, namely by the direct assessment of the presence of tunicamycin within exosomes. This feature can reveal new protective phenomena in which cancer cells are able to detoxify high concentrations of cytoplasmic drugs by exosomal release. In terms of THP-1 modulation, it is also worth to further investigate which are the mechanisms underlying the *IL8* upregulation in THP-1; since it is a parallel characteristic of T-47D cells and *IL8* mRNA is also present in exosomes. Additionally, it still unclear and interesting to analyse the differential proteomics of breast cancer cells exosomes, ER stressed and unstressed.

To conclude, it will be of great interest to evaluate how glucose/lactate metabolism occurs within the tumor. Tumor cells seem to be highly dynamic adapting their metabolism to the microenvironment features.

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