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ER stress, autophagy and immunogenic cell death in Photodynamic Therapy-induced anti-cancer immune responses

Abhishek D. Garg[1,*] and Patrizia Agostinis[1,*]

Mailing addresses:

[1]Cell Death Research & Therapy (CDRT) Unit, Department for Cellular and Molecular Medicine, University of Leuven (KULeuven), Leuven, Belgium;

Correspondence:

*Prof. Patrizia Agostinis; Cell Death Research and Therapy Unit, Dept. Cellular and Molecular Medicine, Campus Gasthuisberg, O&N1, Herestraat 49, Box 802, 3000 Leuven, Belgium;

Email: patrizia.agostinis@med.kuleuven.be; Telephone: +32 16 330650; Fax: +32 16 345995

*Dr. Abhishek D. Garg; Cell Death Research and Therapy Unit, Dept. Cellular and Molecular Medicine, Campus Gasthuisberg, O&N1, Herestraat 49, Box 802, 3000 Leuven, Belgium

Email: abhishek.garg@med.kuleuven.be and abhishekdgarg@gmail.com; Telephone: +32 485983342; Fax: +32 16 345995

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Abbreviations: CD – cluster of differentiation; CMA – chaperone-mediated autophagy; CRT – calreticulin; DAMP – damage-associated molecular pattern; Ecto – surface exposed or surface tethered; ER – endoplasmic reticulum; HMGB1 – high-mobility group box 1; HSP – heat-shock protein; Hyp- hypericin; ICD – immunogenic cell death; IFN – interferon; IL – interleukin; NO – nitric oxide; PDT – photodynamic therapy; Phox – photo-oxidative; ROS – reactive oxygen species; TLR – toll-like receptor;

Abstract

Tumours are a form of pseudo-organs with their own microenvironment where the cancer cells nurture a dysfunctional immune environment incapable of inciting anti-tumour immunity. It had been proposed that the only way to counteract such an immune system dysfunction in tumours is by eliciting, therapeutically, a cancer cell death pathway that is accompanied by high immunogenicity and possibly inhibits or reduces the influence of the pro-tumourigenic cytokine signalling. Subsequently, a small and a large-scale screening study as well as several targeted studies found that few, selected anticancer therapeutic regimens are able to induce a promising kind of cancer cell demise called immunogenic cell death (ICD), which can activate immune system owing to the spatiotemporally defined emission of danger signals. Recently, photodynamic therapy (PDT) utilizing the photosensitiser, hypericin (Hyp), became the first PDT paradigm characterized to be capable of inducing *bona fide* ICD. In the present perspective, we discuss the various technical, conceptual, molecular advancements and unprecedented results revealed by Hyp-PDT that have influenced the fields of ICD, ER stress biology, cancer cell death, anti-cancer immune responses, photoimmunology and PDT.

Introduction

The archaic view that tumours are insular masses composed of proliferating cancerous or tumourigenic cells only has been long disapproved.[1] It is well acknowledged now that tumours are a form of pseudo-organs with their own microenvironment composed of a diverse array of cells apart from tumour cells like pericytes, myofibroblasts, fibroblasts, endothelial cells, bone marrow-derived vascular progenitor cells, mesenchymal stem cells and various types of innate/adaptive immune cells.[1] This microenvironment is shaped by various physicochemical (hypoxia; acidosis; redox modifications) and biological processes (factors secreted by tumour cells, stromal cells and immune cells; lymphatic and endothelial vascular biology). In fact, a clinically-relevant tumour is an immune organ in its own right since cancer cells in such a tumour create and sustain a characteristic immune microenvironment.[2] Such a microenvironment is composed of predominantly immune cells mediating immunosuppression or tolerogenicity and few immune cells sustaining immunosurvelliance (especially at the peripheral or proliferative margins of the tumours). These few latter cells though fail to incite stable anti-tumour immunity, due to the absence of proper immunostimulatory factors (like danger signals or agonists of toll-like receptors or TLRs).[2, 3] Thus, cancer cells nurture a dysfunctional immune microenvironment incapable of inciting anti-tumour immunity. Cancer

cells accomplish this by exhibiting low immunogenicity, by dying through non-immunogenic or tolerogenic cell death pathways following therapeutic treatment (which further helps in maintaining low immunogenicity), by secreting tumour-promoting or immunosuppressive cytokines (mainly under the control of a transcriptional program regulated by NF-kB, STAT3 or AP-1), by producing enzymes or ligands that inhibit anticancer immunity (e.g. indoleamine-2,3-dioxygenases and programmed cell death-ligand 1 or PD-L1) and by inducing immune cell polarizations that also result in secretion of immunosuppressive or pro-tumourigenic cytokines (encouraged in part by tumour-associated 'mild but chronic' ER stress and cancer cell or stromal cell-associated autophagy).[2, 4-7]

Based on the above observed characteristics, it was proposed that the only way to counteract such an immune system dysfunction in tumours is by eliciting, therapeutically, a cancer cell death pathway that is accompanied by high immunogenicity[8] and possibly inhibits or reduces the influence of the pro-tumourigenic cytokine signalling.[9, 10] At first this was considered to be a tough task since most anticancer therapeutic regimens tended to induce cancer cell death (like physiological apoptosis) that was non-immunogenic, low-immunogenic or tolerogenic.[8, 11] However over last few years, a small[12] and a large-scale screening study[13] as well as several targeted studies[9, 14] have found that few, selected anticancer therapeutic regimens are able to induce a promising kind of cancer cell demise called immunogenic cell death (ICD).[8]

ICD, as opposed to its 'kin' the non-immunogenic or tolerogenic cell death pathway (i.e. physiological apoptosis), can activate immune system owing to the spatiotemporally defined emission of damage-associated molecular patterns (DAMPs) that act as danger signals.[8] Under normal conditions, molecules that can act as DAMPs have mainly non-immunology related functions inside the cell. However, under stressful, damaging or injuring conditions, these molecules or DAMPs become exposed either in a surface-associated or extracellulary secreted manner by the cell.[7, 14, 15] In the extracellular space these DAMPs might orchestrate immunostimulatory activity thereby acting as danger signals. Such DAMPs are capable of exhibiting various kinds of danger signal-activities e.g. activation of TLR signalling, enhancement of opsonisation (by interacting with scavenging receptors, phagocytic receptors or complement proteins), activation of inflammasome complex (by interacting with purinergic receptors), activation of specific polarization or differentiation states in immune cells, acting as cytokines and chemoattractants and last but not least, positive regulation of general anti-tumour immunity effector processes.[7-11, 14] Within the context of anticancer immune responses, DAMPs allow efficient communication of the 'antigenic pattern' of dying cancer cells to the host immune cells and thereby pave way for eliciting potent anti-cancer immunity.[8, 11]

DAMPs or danger signals found to be crucial for ICD consist of – (a) chaperone DAMPs e.g. surface exposed calreticulin (ecto-CRT),[12, 16] ecto-HSP90[17] and/or ecto-HSP70; (b) nucleotide DAMPs e.g. actively secreted ATP[8] and (c) endokine or chaperokine DAMPs: endogenous cytokines and chaperones performing cyto-/chemo-kinetic functions can be released by dying cells e.g. high mobility group box 1 (HMGB1), an endokine, and chaperokines like CRT, HSP70, HSP90.[8, 10, 14] These respective DAMPs emitted by ICD, in turn bind to their respective (cognate) receptors or targets on immune cells thereby activating a cascade of beneficial effects (demonstrated mainly for chemotherapy-induced ICD). Ecto-CRT binds to scavenging/phagocytic receptors on dendritic cells (DCs) and acts as an 'eat me' signal by facilitating engulfment of cell debris acting as a source of cancer antigens. Ecto-HSP90/HSP70 in various settings help mediate immunogenicity although their ability to directly mediate phagocytosis is still debatable. Secreted ATP stimulates the purinergic P2RX7 receptors on the DCs thereby activating the NLRP3 inflammasome-mediated maturation and subsequent release of IL-1β such that this cytokine further helps in functional polarization of IFN-γ producing cytotoxic αβT cells and stimulates IL-17 production from Th17 γδT cells. Moreover secreted ATP also helps in recruitment and local differentiation of the $CD11c^{+}CD11b^{+}Ly6C^{hi}$ myeloid cells (by binding to P2Y2 receptors on their surface), which are crucial for chemotherapy-induced anticancer immunity. Last but not least, extracellulary released HMGB1 acts as a TLR4 agonist on DCs and thereby facilitates a MyD88-dependent pathway crucial for proper antigen processing.[8, 10, 14, 17] It is worth mentioning here that, chaperokines like extracellulary released HSP70 can also act as potent TLR4 agonists.[8, 10, 14, 17] For a more comprehensive discussion on ICD, DAMPs and danger signalling, please refer to certain recent reviews.[5, 8-11, 14]

ICD tends to exhibit a high exclusivity when it comes to therapeutic induction in cancer cells because only a handful of modalities are capable of inducing it. For a detailed description of currently known ICD inducers, please refer to certain recent reviews.[8, 9, 14] Such an exclusive inducer-induction relationship exists because ICD requires, as pre-requisite, induction of reactive oxygen species (ROS)-based endoplasmic reticulum (ER) stress, where the ROS may or may not be mainly ER-directed.[11, 18] The concomitant production of ROS and the resultant induction of ER stress are vital for elicitation of the danger signalling pathways responsible for the intracellular trafficking and subsequent surface/extracellular emission of DAMPs/danger signals.[11, 18] It is this exclusivity that makes every inducer of ICD, a special anticancer therapeutic modality.

Recently, photodynamic therapy (PDT) utilizing the photosensitiser, hypericin (Hyp), became the first PDT paradigm characterized to be capable of inducing ICD.[10, 14, 19-21] Moreover, Hyp-PDT exhibits several properties which currently define an 'ideal ICD inducer'.[10, 14] From the perspective of PDT-based photo-

immunology,[15, 22, 23] it is an important achievement for Hyp-PDT to be able to join an exclusive club of assorted therapies inducing ICD. More importantly, Hyp-PDT has actually helped in technical as well as conceptual advancement of the ICD and danger signalling concepts (Fig 1).[9, 10, 14] Besides these advancements, the usage of Hyp-PDT has also helped unravel a diversity of unprecedented results (Fig 1). For example, this research has helped in delineating PERK, an ER sessile sensor of ER stress, as a master regulator of ICD and ER stress molecular cell biology.[24] It has also helped in revealing the context-dependent role of cancer cell-associated autophagy in regulating the interface between dying cancer cells and the immune system (Fig 1).[25-27]

In the present review, we discuss the various technical, conceptual, molecular advancements and unprecedented results revealed by Hyp-PDT (Fig 1), that have influenced the fields of ICD, ER stress biology, cancer cell death, anti-cancer immune responses, photoimmunology and PDT.

Hypericin-based PDT: Evolutionarily destined to induce responses against "non-self" entities?

Hyp-PDT elicited induction of ICD requires an interesting interplay of various biophysical processes, photobiological processes, stress-response signalling and cell death signalling. Hence, before we commence the discussion on Hyp-PDT elicited ICD, we would like to discuss (briefly) the basic processes associated with this modality that make its therapeutic effect possible.

Hyp-PDT initially entails that, the photosensitive drug (or photosensitiser), hypericin, reaches a proper sub-cellular localization so that the pivotal pre-requisite of ICD can be induced i.e. ROS-based ER stress.[15, 23, 24, 28, 29] Hypericin is considered to be one of the most powerful naturally occurring photosensitisers, which when added to target cells, can predominantly accumulate within the membranes of the ER.[29] The exact mechanism behind this is elusive however there are indications that the affinity of hypericin for phosphatidylcholine[30-32] might be a critical factor behind its preferential ER membrane localization. This is because the cellular concentrations of phosphatidylcholine tend to escalate as one goes from the plasma membrane towards the ER. In fact, the ER membranes boast of the highest final phosphatidylcholine concentration as compared to other cellular membranes.[33] This conjecture is comforted by the observation that, SERCA2, the ER sessile protein preferentially photo-damaged by Hyp-PDT[34] tends to reside in a phosphatidylcholine-rich environment.[35] Once localized in the ER, hypericin can then be activated by visible light of a particular wavelength (e.g. 595 nm) thereby leading to its photoexcitation – a state from which it

strives to escape and come back to ground state.[29] This process of coming back to ground state causes ROS production when hypericin reacts with cellular components in the presence of oxygen.[29] Hypericin when photoactivated has a high triplet quantum yield and ability to produce considerable amounts of singlet oxygen.[29] Now, since such ROS species have limited half-life and restricted diffusion range,[22] they end up generating most of their biological effects wherever they were originally produced, which in case of Hyp-PDT, is the ER.[19, 24, 34] All this leads to ROS-based ER stress or photo-oxidative (phox)-ER stress induced by Hyp-PDT.[9, 19, 21] The signalling pathways behind these phenomena are briefly discussed in the next section. It is noteworthy that Hyp-PDT is capable of inducing signatures of phox-ER stress not only *in vitro* but also *in vivo*, within a treated-tumour.[19, 24, 28]

Of note, hypericin is an important secondary metabolite derived from most of the plant species belonging to the genus, *Hypericum*. *Hypericum perforatum* (or St. John's wort) is the most common representative of this genus.[29] Curiously, it has been postulated that hypericin may participate in anti-pest defence responses in plants (Fig 2).[29] More specifically it has been reported that when pests attack plants, a combination of pest-derived elicitors (so called 'herbivore-associated molecular patterns') and DAMPs released by (pest-induced) damaged plant cells can cause activation of a defence response (Fig 2).[36] The aim of this defence response is two-sided. On one hand it aims to activate direct anti-pest mechanisms (e.g. consisting of hypericin release in *Hypericum* plants[29, 37]) and wound healing responses while on the other, it aims to alert other not-yet-damaged plant cells about the impending pest-related danger (Fig 2).[36] Such a defence response is mainly mediated by jasmonic acid (amongst other metabolites) – which regulates wound healing responses, danger signalling and direct or indirect anti-pest defence responses.[36, 38] Interestingly, the production of jasmonic acid in response to plant-derived DAMPs can be further enhanced by addition of ATP, an important danger signal.[38] In case of *Hypericum* plants specifically, it has been found that jasmonic acid can elicit the production of hypericin in undamaged plant cells[37]. This hypericin (possibly packaged in impermeable pigment vesicles[29]) can be released upon pest afflicted-damage thereby causing a light-mediated killing of pests (*de facto* naturally executed PDT-mediated pest killing) (Fig 2).[29] All this, taken together with the currently known ICD-inducing effect of Hyp-PDT (described later), suggests that Hyp-PDT might represent a modality that is evolutionarily destined to activate immunological or defence responses against "non-self" entities.

ICD and danger signalling induced through 'focused' ROS-based ER stress: A technical advancement

Most of the ICD inducers characterized before (or even after Hyp-PDT was found to induce ICD) triggered cancer cell death and danger signalling *via* relatively mutually exclusive targets.[10, 21] More specifically, while danger signalling was triggered *via* ER-based targeting yet cell death signalling was triggered *via* non-ER associated targets. Such targets included, DNA replication/repair machinery components (as applicable to anthracyclines,[12, 16] mitoxantrone,[12] oxaliplatin,[18] UVC irradiation,[18] radiotherapy[12] and cyclophosphamide[39]), certain cytosolic components (as applicable to shikonin[40] and bortezomib[17]) and certain cell surface-associated components (as applicable to 7A7[41] and cardiac glycosides[13]).[9, 10, 14, 21] Thus for all such inducers, these non-ER associated sites represented the site of 'focused' effect while ER represents as site of 'collateral' effect.[9, 10, 14] Accordingly, all these agents were classified as Type I ICD inducers, *i.e.* modalities that induce pro-death signalling through non-ER associated targets but induce ICD-associated danger signalling through collateral ER stress effects.[9, 10, 14, 21] Type I ICD inducers have several advantages but their tendency to induce ICD *via* collateral ER stress is their "Achilles' heel". This is because the collateral nature of this effect ensures that the ICD-associated danger signalling is complex, regulated in a highly multifactorial manner and susceptible to therapy-resistant microevolution in cancer.[9, 10, 14, 21] Thus Type I ICD inducers, in principle, did not exploit ICD and its potential to the fullest extent.

Discovery of Hyp-PDT induced ICD helped characterize the first Type II ICD inducer i.e. a modality that selectively targets the ER to induce both pro-death signalling as well as ICD-associated immunogenicity in an ER-focused manner (Fig 1).[9, 10, 14, 21] Basically the induction of phox-ER stress by Hyp-PDT induces one main danger signalling arm and three pro-death signalling arms.[19, 24, 28] Interestingly, following Hyp-PDT, both danger and pro-death signalling seem to be originating from the ER sessile stress sensor molecule – PERK; thereby making PERK the master regulator of cell death, immunogenicity and secretory pathway (discussed in the next section).[19, 24, 28] The regulation and mechanics of the danger signalling arm is discussed in details, in the next section. As far as the three pro-death signalling arms are concerned, PERK is at the 'core' of all three.[24, 28] In case of the first arm, phox-ER stress elicited by Hyp-PDT activates the unfolded protein response (UPR) signalling cascade.[21, 24] The aim of the UPR is two-faceted - it strives to restore ER homeostasis and promote cellular survival if the ER stress is mild. However if ER stress is severe beyond the "point-of-no-return", the UPR tends to elicit pro-death signalling.[42] Phox-ER stress induced UPR is characterized by up-regulation of various ER chaperones, up-regulation of UPR-associated transcription factors, activation of the PERK branch and activation of the IRE1α branch.[24, 28] However, it is the PERK branch (PERK-P → eIF2α-P → ATF4↑) which under phox-ER stress, activates the pro-apoptotic

transcription factor CHOP, which in turn predominantly mediates the Hyp-PDT induced mitochondrial apoptosis.[24, 28] We recently reported that it might not be the CHOP-Bim arc that is behind mitochondrial apoptosis for Hyp-PDT.[28] Instead we hypothesized that possibly a rather enigmatic and only recently characterized process of cell death induced by ATF4/CHOP-based increase in protein synthesis and oxidative stress may underlie Hyp-PDT induced apoptosis.[28] On the other hand, in the second arm, PERK regulates the expression of the pro-apoptotic BH3-only protein, Noxa (accompanied by down-regulation of the anti-apoptotic BH3-only protein, Mcl-1). Here, Noxa helps in further amplification of pro-apoptotic signalling independent of CHOP signalling associated with the first arm.[28] Last but not least; in case of the third arm the cell biology of PERK is crucial for the propagation of pro-death signalling. More specifically, PERK is required at the mitochondria-associated membranes (MAMs) so as to physically tether the ER to a fraction of mitochondria.[24] In this set-up, following phox-ER stress, PERK promotes the transfer of ROS created at the ER to the mitochondria thereby predisposing the pool of mitochondria in close contact with the ER to full blown mitochondrial apoptosis.[24] The complex interplay of these three pro-death arms, master regulated by PERK, ultimately culminates into Bax/Bak mediated mitochondrial apoptosis characterized by mitochondrial depolarization, cytosolic cytochrome c release and caspase-3 activation.[24, 28]

'Focused' ROS-based ER stress induced by Hyp-PDT instigates potent cancer cell death as well as elicits high immunogenicity thereby leading to ICD (Fig 3).[9, 14, 21] More specifically, Hyp-PDT induces in the pre-apoptotic stage; the active emission of three crucial danger signals i.e. ecto-CRT, ecto-HSP70 and secreted ATP – all of which are *bona fide in vitro* markers of ICD (Fig 3).[19, 20] Here, pre-apoptotic stage is a cellular stage devoid of any apoptotic signs including phosphatidylserine externalization following treatment. This is followed by passive release of chaperokines like HSP70 and HSP90 in the late apoptotic phase (i.e. during secondary necrosis) (Fig 3).[19, 20] Hyp-PDT induced ICD represents the first cell death modality described to be associated with simultaneous emission of three danger signals.[19-21] Moreover Hyp-PDT was found to be superior to chemotherapy (mitoxantrone or doxorubicin), in terms of the numbers (three), overall amounts (2-3 folds higher) and speed of the emission (within 15-30 mins post-treatment) of danger signals exposed during the pre-apoptotic stage.[19-21]

These *in vitro* observations are also strongly supported by *ex vivo* and *in vivo* immunological experiments (Fig 3). Hyp-PDT treated cancer cells are recognized as well as phagocytosed efficiently by innate immune cells (e.g. Mf4/4 murine macrophages, JAWSII murine dendritic cells or DCs and human primary monocyte-derived DCs), more than untreated cancer cells.[19] In fact also for Hyp-PDT treated cancer cells, ecto-CRT acted as an 'eat me' signal such that anti-CRT antibodies reduce the phagocytosis of treated cells.[19, 20]

Following these phagocytic interactions, DCs interacting with Hyp-PDT treated dead/dying cancer cells are efficiently stimulated and exhibit properties of fully mature immunogenic DCs.[19, 25, 43] More specifically, the interacting DCs exhibit phenotypic maturation ($CD80^{high}$ $CD83^{high}$ $CD86^{high}$ $MHC\text{-}II^{high}$) as well as immunogenic functional stimulation (NO^{high} $IL\text{-}10^{absent}$ $IL6^{high}$ $IL1\beta^{high}$ $IL12p70^{medium}$) (Fig 3).[19, 25] Thus, DCs fed with Hyp-PDT treated cancer cells not only exhibit cytokine signaling stimulation but also show signs of respiratory burst (as characterized by the presence of NO or nitric oxide) – a unique observation.[19, 25] We suspect that the high ecto-HSP70 induced in Hyp-PDT treated cancer cells[20] might be behind this DC-based respiratory burst since it was recently shown for PDT that ecto-HSP70 up-regulates immune cell-derived NO (*via* TLR2 signaling).[44] It is also noteworthy that the DC-derived IL10 is absent for Hyp-PDT induced ICD (across human bladder carcinoma as well as human melanoma cells).[19, 25] This is a very unique finding since even for chemotherapy-mediated ICD IL10 has not been reported to be strongly absent.[45] This is also important for increasing the potency of ICD since it was recently shown that activation of TLR signaling one of the pre-requisites for ICD is strongly down-regulated by IL10 (*via* microRNA-146b).[46] This clearly shows that cancer cells undergoing Hyp-PDT-induced ICD form a highly productive interface with the DCs,[43] which is better than chemotherapy-induced ICD.

The fully mature immunogenic DCs elicited by cancer cells undergoing Hyp-PDT induced ICD[43] go onto stimulate the anticancer mode of the adaptive immunity (Fig 3).[19, 25, 27] More specifically, these fully mature immunogenic DCs induce efficient proliferation and clonal expansion of IFNγ producing $CD4^+$ and $CD8^+$ T lymphocytes – a *bona fide* signature of stimulation of anticancer immunity (Fig 3).[25, 27] In fact, this anticancer immunity induced by Hyp-PDT elicited ICD *in vitro* is capable of efficiently rejecting tumors *in vivo* – a signature of antitumor immunity (Fig 3).[19, 21] Immunization with cancer cells undergoing Hyp-PDT induced ICD (in a prophylactic syngeneic murine model)[19] or *in vivo* treatment of tumors with Hyp-PDT (in a curative syngeneic murine model)[47] causes efficient rejection of tumors upon re-challenge with transplantable tumor cells. It is noteworthy that, Hyp-PDT is capable of inducing signatures of ER stress, a crucial pre-requisite of ICD, in a treated tumor *in vivo*[28] – a further evidence towards novelty of this 'focused' ROS-based ER stress eliciting modality (Fig 1).

Taken together all these observations clearly show that 'focused' ROS-based ER stress elicited by Hyp-PDT is capable of inducing *bona fide* ICD associated with pre-clinically relevant anticancer immune responses (Fig 3).[10, 14, 19, 21] More importantly this ICD subroutine is visibly better and technically more unique than those induced by radiotherapy, oncolytic virotherapy, various chemotherapeutics or targeted therapeutics[5, 9, 10, 14] – an argument that carries high clinical relevance for Hyp-PDT in near future.

Revealing the plasticity in danger signalling during ICD: A conceptual advancement

Danger signals as molecular entities had been around for a long time but the signalling pathways behind their active emission (i.e. in the absence of plasma membrane permeabilization) dubbed as danger signalling pathways had been seldom analysed. With time however studies on danger signalling started to be published and of the known ICD-associated DAMPs, pathways induced by chemotherapy and Hyp-PDT treatment for emission of ecto-CRT and secreted ATP emerged.[8, 10, 18, 21] Surprisingly the chemotherapy-induced danger signalling pathways were found to be highly complex and heavily multi-factorial.[8, 10, 14, 18, 19] Unfortunately the complexity and multi-factorial nature made these chemotherapy-induced danger signalling pathways highly susceptible to therapy-resistant microevolution in cancer.[5, 10, 14] A data mining and bioinformatics analysis published recently showed that indeed chemotherapy-induced danger signalling pathways were susceptible to subversion in cancers (e.g. through mutation or ablation of caspase-8 and eIF2α-P, important danger signalling mediators).[10] In contrast however, Hyp-PDT-induced danger signalling pathways were comparatively less susceptible to subversion by cancer cells (Fig 1).[5, 10, 14]

Hyp-PDT induced pre-apoptotic ecto-CRT through a conglomeration of processes consisting of ROS-based ER stress, PERK-mediated proximal secretory pathway, ER-to-Golgi transport, Bax/Bak, classical secretory pathway, PI3K-mediated plasma membrane trafficking and LRP1-based surface docking.[19, 21] Similarly, Hyp-PDT induced ATP secretion followed a highly overlapping pathway for trafficking consisting of every regulator applicable to Hyp-PDT induced ecto-CRT except Bax/Bak.[19, 21] This is in stark contrast to the pathways for chemotherapy such that Hyp-PDT did not require a number of components essential for chemotherapy-induced danger signalling (e.g. ERp57, caspase-8, eIF2α-P and lipid rafts).[10, 19-21] For a more comprehensive comparison of chemotherapy and Hyp-PDT induced danger signalling pathways, please refer to recent comprehensive reviews.[8, 10, 14, 21] These trends clearly show that the danger signaling cascades trafficking DAMPs exhibit an ICD-inducer dependent plasticity. Such that mainly chemotherapeutic agents (i.e. Type I ICD inducers like mitoxantrone or oxaliplatin) induce relatively complex pathways while Hyp-PDT (a Type II ICD inducer) elicits a much simpler and consolidated version.[14] Moreover, it is also noteworthy that the ability of Hyp-PDT induced ecto-CRT to be trafficked in a caspases or caspase-8 independent manner is the first instance supporting uncoupling of cell death and danger signaling pathways for an ICD paradigm, *in vitro* and *in vivo*.[19, 21]

Last but not least, these observations also support the conjecture that ICD induced by 'focused' ROS-based ER stress is superior to that induced by 'collateral' ROS-based ER stress (Fig 1).[10, 14, 21]

Autophagy as an immunoevasive tactic employed by cancer cells to resist ICD: An unprecedented advancement

There is significant plasticity in danger signalling and the immunoeffector properties associated with ICD depending on the therapeutic modality under consideration. However, one basic pre-requisite has been found to be common amongst all discovered ICD inducers i.e. ER stress (either caused by ROS or accompanied by ROS production at the ER).[14] ER stress is henceforth a general 'enabler' of ICD. On the other hand, the most apical or innate aim of ER stress-associated stress signalling is usually to rescue cellular homeostasis and promote pro-survival[42] – an aim partly supported by concomitant induction of macroautophagy[48] (hereafter referred to as simply – autophagy). For most ICD inducers reported so far (including Hyp-PDT), autophagy accompanying cell death has been found to impede pro-death signalling since therapy-induced autophagy helps in recycling of damaged subcellular targets and restore nutrient and metabolic health thereby assisting in cytoprotection.[48-50] Thus, not surprisingly researchers became curious about the ER stress-autophagy connection in the ICD paradigm.

For chemotherapy-induced ICD, researchers reported that autophagy, like ER stress, acted as 'enabler' of ICD by assisting in ATP secretion and *bona fide* elicitation of anticancer immune responses, *in vitro* and *in vivo*.[51] Thus the ER stress-autophagy link was implicated as a general 'enabler' of ICD, proposed to be even applicable to Hyp-PDT[11] – a point that needed urgent verification.

Chemotherapy induced autophagy tends to be predominantly mitophagy-based response (i.e. autophagy for mitochondria) and in many cases instigated by p53 due to DNA-associated damage induced by these therapeutics.[52] On the other hand, Hyp-PDT activates an autophagic paradigm that literally 'chases' photo-oxidative damage induced by this modality. As described above, Hyp-PDT entails instigation of an initial 'severe' ROS-based ER stress which subsequently culminates into a combination of ROS-based mitochondrial stress and general apoptosis-associated mitochondrial depolarization.[27] Simultaneously, Hyp-PDT initially activates a predominant reticulophagy-based response (i.e. autophagy for ER) which ultimately culminates into predominant but not exclusive mitophagic response.[53] Thus clearly the biochemistry of autophagy induced by these two respective modalities is very different[27] – a point we envisaged could lead

to unprecedented observation about the role of cancer cell-associated autophagy in ICD and anticancer immunity regulation.

Hyp-PDT induces ER stress due to elicitation of considerable proteotoxicity (i.e. accumulation of oxidatively damaged proteins) in cancer cells such that ablation of autophagy (*via* ATG5 knock-down or $ATG5^{KD}$) causes further increases in proteotoxicity and ER stress.[25] The latter is characterized by increased expression of the chaperone – BiP/GRP78, temporally sustained increase in eIF2α-P and increased up-regulation of the pro-apoptotic transcription factor CHOP in different cancer cell types (Fig 4). Curiously, we found that following Hyp-PDT, autophagy suppress the induction of ecto-CRT (without affecting ATP secretion) such that $ATG5^{KD}$ causes approximately 2-fold increase in ecto-CRT thereby reflecting a strong correlation between levels of proteotoxicity, levels of ecto-CRT, the amount of ER stress.[25] Theoretically, based on these observations and the known mechanisms of Hyp-PDT, one can conclude that Hyp-PDT treatment causes ROS production at the ER, which causes ER stress as well as ER-associated proteotoxicity. This combination of ER stress and ER-associated proteotoxicity might be behind the ability of CRT to "escape" the ER and get translocated towards the cell surface.[25] Thus any process that augments proteotoxicity (e.g. $ATG5^{KD}$) should augment ecto-CRT and *vice versa*. Moreover, $ATG5^{KD}$ in Hyp-PDT treated cancer cells also has functional consequences on immunological determinants of ICD and anticancer immunity.[25] $ATG5^{KD}$ in cancer cells undergoing Hyp-PDT induced ICD causes increased phenotypic maturation of DCs, increased DC-derived IL-6 production and augmentation of DC-mediated clonal expansion of (IFN-γ producing) $CD4^+/CD8^+$ T cells.[25] Thus, Hyp-PDT-induced autophagy in cancer cells plays an unprecedented immunoevasive role by suppressing the immunogenicity of dying cancer cells and diminishing processes crucial for elicitation of anticancer immunity (Fig 1).[25, 27]

These observations raise several corollary questions e.g. is this immunoevasive behaviour of autophagy specific for Hyp-PDT? Is this behaviour specific for the cancer type used in the corresponding study i.e. melanoma? And last but least, are these results specific for macroautophagy or other mammalian autophagic pathways can also affect immunogenicity? All of these questions require further targeted analysis but there are already certain indications that may suggest a preliminary pattern. Firstly, it has been shown that macroautophagy can also play an immunoevasive role in two other paradigms apart from Hyp-PDT[25] i.e. treatment with hypoxia[54] and cancer cell-specific ablation of autophagy-essential gene, FIP200[55] – which means that the above results may have a certain level of general implications. Of note, FIP200 or focal adhesion kinase family interacting protein of 200 kDa is a part of the Unc51-like kinase 1 (ULK1) complex (also consisting of other proteins like ULK1/2, ATG13 and ATG101).[48] The ULK1 complex is a chief

initiator of autophagosome formation in mammalian systems.[48] Secondly, while the Hyp-PDT study was done with melanoma as a model system the studies with hypoxia[54] and FIP200 ablation[55] were in fact carried out in lung cancer[54] and breast cancer models[55] respectively thereby meaning that autophagy's immunoevasive behaviour might not be cancer type-specific but rather treatment/stress-type specific.

Autophagy is in principle, a non-specific bulk degradation pathway utilized by the cells for nutrient turn-over and damage-mitigation.[56] Apart from this, mammalian cells also exhibit two additional autophagic pathways i.e. microautophagy and chaperone-mediated autophagy (CMA).[56] Microautophagy involves non-selective lysosome-based direct engulfment of cytoplasmic cargo *via* lysosome-membrane based invagination, protrusion/septation and vesicle scission into the lumen.[57] On the other hand, CMA is a selective form of autophagy where the cytosolic chaperone heat shock cognate 70 (Hsc70) binds damaged proteins possessing a motif identical or similar to the pentapeptide KFERQ and helps transfer these proteins across the lysosomal membranes and into the lysosomal lumen for degradation.[58] The process of protein translocation across lysosomal membrane is facilitated by the CMA-essential receptor, lysosome-associated membrane protein 2A (LAMP2A).[58] Not much is known about the role of cancer associated microautophagy and CMA in regulating anticancer immunity. However, we recently observed that fibroblasts lacking the CMA-essential gene LAMP2A are unable to emit ecto-CRT after chemotherapy (i.e. mitoxantrone) or Hyp-PDT treatment while the ability to secrete ATP is retained.[26] It has been reported that cells lacking CMA exhibit compensatory rise in macroautophagy[58] thereby raising the possibility that this increased macroautophagy may ablate ecto-CRT induction in cells lacking LAMP2A.[25] However contrary to this hypothesis, knocking down macroautophagy (*via* $ATG5^{KD}$) failed to restore ecto-CRT following chemotherapy/Hyp-PDT.[26] These results raise many interesting possibilities and incentive for further research. On the face of it, these results suggest that CMA can regulate danger signalling[26] – a very interesting possibility. However this assumption is based on the fact that macroautophagy is the only compensatory mechanism up-regulated in cells lacking LAMP2A. What if microautophagy is also upregulated in CMA-deficient cells and might be ablating ecto-CRT? Studies have shown that the CMA-essential chaperone Hsc70 might play a dual role when it comes to assisting in catabolic processes.[59] Researchers recently showed that microautophagy, which unfolds independently of macroautophagy regulators (like ATG7) and LAMP2A is actually dependent on the activity of Hsc70.[59] Here, Hsc70 is capable of guiding the KFERQ-motif containing proteins to late endosomes or multi-vesicular bodies (MVBs) for subsequent microautophagy-like degradation.[59] Thus, it is of outmost importance to ascertain

the effect of interplay between macroautophagy-CMA-microautophagy under stress on danger signalling and, in long run, anticancer immunity.

ROS-based ER stress through Hyp-PDT: A "near-to-ideal" ICD inducer?

ICD is an emerging process and new ICD inducers are being added to the list on nearly a quarterly basis.[10, 14] However, this also means that more scrutiny is required as to what is expected off an ICD inducer in terms of efficiency of anticancer cytotoxic and immunological effects. To this end, we had recently proposed an initial list of characteristics that an ideal ICD inducer should have[10, 14] i.e., ability to induce efficient programmed cell death; capability to induce strong immunogenicity; non-susceptibility to drug-efflux channels; capacity to induce severe focused ER stress; capability to overcome loss-of-function mutations that cripple danger signalling; capability to down regulate cancer-based induction of pro-inflammatory transcription factors; capacity to exert negligible inhibitory effects on anti-tumorigenic immune cells; capacity to exert inhibitory effects on pro-tumorigenic immune cells and last but not least capacity to directly target metastasized cells.[10, 14] Hyp-PDT ticks all the boxes across this board[10, 14] except two; firstly, the effects of Hyp-PDT on tumour-associated pro-tumorigenic immune cells are not yet known. Secondly, due to the peculiar nature of the photodynamic treatment paradigm (requiring the combined availability of drug, oxygen and light targeting the tumour cells), just like radiotherapy, it cannot target the metastasized cells directly. While more research can help answer the former point, the latter point is more complex to address. It is noteworthy that, all the thus-far characterized photosensitizers are incapable of directly targeting the metastasized cancer cells within a PDT paradigm[60] However, like radiotherapy, Hyp-PDT might exploit "abscopal effects" for anticancer immunity based elimination of deeper (inaccessible) tumour cells and distant metastasis.[61] Hyp-PDT is capable of inducing ER stress *in vivo*,[28] ICD *in vivo* and *in vitro*[19, 21, 25] and has been shown to induce clinically-beneficial tumour regressive effects in the few clinical trials carried out.[9] This means that there is a distinct possibility that Hyp-PDT can induce "abscopal effects" – a conjecture that needs urgent attention. Nevertheless, Hyp-PDT has more ideal properties of ICD inducer than most other inducers[10, 14] and hence it is most definitely a near-to-ideal ICD inducer that needs to be exploited preclinically and clinically.

Apart from the photosensitiser function of hypericin it has also been found that in the absence of light irradiation (i.e., in the absence of photogenerated ROS or non-PDT conditions) hypericin may exhibit some ROS-independent effects, in a concentration-dependent fashion.[62] In non-PDT conditions, supra-high

concentrations of hypericin (mostly in μM ranges i.e. ~3 to ~420 μM) have been found to inhibit dopamine β–hydroxylase enzyme or the kinase activity of protein kinases A/C.[62] However, we believe that these ROS-independent effects (dark-activity) of hypericin have little implications for Hyp-PDT induced ICD. This is because; firstly, photogenerated ROS produced by light activation of hypericin concentrations (100-200 nM)[19, 20, 25, 26] - that are considerably less than the concentrations required for non-specific dark-effects- have been shown to be required for the activation of pathways leading to the emission of ICD-associated DAMPs. Secondly, we have observed that the kinases known to be relevant for ICD are either functionally not affected (e.g. PI3K[63]) or even stimulated (e.g. PERK[19, 24, 28]) following Hyp-PDT, thus indicating the ability of Hyp-PDT to propagate signalling pathways or processes modulating ICD in a light, and ROS-specific manner. These observations confirm that the non-PDT effects of hypericin might not be relevant for ICD. It would be interesting however to confirm this point *in vivo*, in near future.

Future Perspectives: Will PDT still lead the way ahead in the fields of ICD and anti-cancer immunity?

The concept of DAMPs and danger signalling came to light in early 1990s.[64] During that decade and the first half of the 2000s, several DAMPs were characterized including ecto-HSPs and ecto-CRT.[7, 65, 66] The concept of ICD was established firmly by a series of publications between 2005 and 2010.[8, 12, 13, 16-18, 45, 51] Following these trends, in 2010-2011, we had anticipated across three separate reviews that PDT has the potential to lead the way ahead for the fields of ICD and anti-cancer immunity.[7, 15, 23] Until that time, PDT had been shown to be associated with various well-established DAMPs like HSP70, GRP94 and GRP78 and had been shown to induce a certain degree of positive results in anti-cancer vaccination set-ups (both pre-clinically and clinically).[67, 68] Also various researchers had described different instances of PDT having a positive impact with regards to anti-tumour immunity in preclinical animal experiments (and even clinical studies) – a point discussed in several comprehensive reviews.[15, 23, 67, 69-71] For instance, PDT paradigms with various photosensitisers like benzoporphryin,[72] hematoporphyrin derivatives,[73] 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-α (HPPH),[74] Photofrin,[68] Foscan,[74] 5-ALA, mono-L-aspartyl chlorin-e6 and etiopurpurin have been shown to induce various levels of anti-tumour immunity (*ex vivo*, *in vitro*, *in vivo* and even in patients).[74] However, while many of these PDT paradigms have been shown to induce various DAMPs (mostly HSPs) yet none of them have been shown to induce all the necessary molecular determinants of ICD simultaneously i.e. pre-apoptotic calreticulin, early ATP secretion and release of TLR4 agonists like HMGB1/HSP70. Thus, these paradigms have seldom reached the same level that certain other therapies like

anthracyclines and radiotherapy have reached, in terms of inducing ICD as a "side-effect" to the original anti-cancer cytotoxic activity.[8]

Our work, published over the period of last one and a half years,[19, 20, 25, 26] along with certain other publications from independent researchers[47] has put Hyp-PDT at the fore-front of various advancements associated with ICD and ER stress biology e.g., the discovery that multiple DAMPs can be co-emitted in pre-apoptotic stage,[19, 20] danger signalling pathways can be plastic rather than rigid in their execution,[14, 19, 20] PERK can regulate MAMs integrity[24] and that cancer cell-associated autophagy can play a context-dependent role in evasion from ICD and anti-cancer immunity.[25, 26] All these advancements have helped accentuate interest of the general scientific audience in Hyp-PDT (Fig 1). It should be noted however, that other photosensitisers might also be capable of inducing ICD or accentuating cancer immunogenicity (e.g. Photofrin[75]) but their ICD-inducing properties need to be thoroughly and more comprehensively tested. We envisage that photosensitisers that predominantly localize in the ER have the highest probability of inducing *bona fide* ICD.[19, 21]

The ICD-inducing characteristics of Hyp-PDT readily vouch for production of anti-cancer vaccines based on this paradigm, for both pre-clinical testing and possible clinical application. Direct *in vivo*/*in situ* treatment of tumours with Hyp-PDT is also a good option but its application is expected to be limited to superficial tumours readily accessible to light irradiation. Moreover, compared to certain other photosensitisers (e.g. Photofrin) that have an excitation spectra in the more red region of the light spectrum (i.e. beyond 620 nm), hypericin's maximum absorption wavelenght is 595 nm, falling in the orange region of the visible light spectrum.[29, 60] Depending on the superficial neoplasm to be treated, this may be a limitation since red light wavelength tends to penetrate relatively deeper into certain tissues *in vivo*/*in situ* than orange light.[29, 60] This makes *in vivo* treatment with Hyp-PDT relatively more superficial, although for certain promising applications, like in the case of superficial bladder cancer, this may not be a critical factor since 595 nm laser light penetrates readily across these superficial lesions.[76] In future, it would be necessary to explore the possibility of making hypericin-derivatives that retain their ER-localizing capabilities but have maximum absorption wavelength in the red region of the light spectrum.

The obvious question at this point is; following all these great accomplishments can Hyp-PDT still continue to lead the way ahead in the aforementioned broad and specialized fields? We envisage that it still can! Hyp-PDT induced ICD has a great technical and conceptual prowess – that in our opinion hasn't yet been completely characterized. Hyp-PDT has instigated several trends in the field of ICD already e.g. the urgent

need to characterize "ideal" or "near-to-ideal" ICD inducers or ICD inducing combinatorial regimens;[10, 14] the need to induce ER stress-based danger signalling through more primary (rather than secondary or tertiary) ER-directed effect;[14, 21] and, the need to further characterize the full-extent of the complexity and plasticity of danger signalling.[5, 14] In fact recently researchers, partly influenced by the complexity of danger signalling revealed by Hyp-PDT, revisited the danger signalling instigated by chemotherapy and went onto reveal that during chemotherapy-induced ICD, ecto-CRT induction can be further accentuated through autocrine or paracrine signalling involving cancer cell-derived chemokines like CXCL8 (in human system) or CXCL2 (in mice system), induced by the chemotherapeutic ICD inducers.[77]

To this end, we envisage that Hyp-PDT can be further used to not only revisit and revise the existing paradigms of ICD and danger signalling but also to unravel and establish new, as-yet-unknown, characteristics of these beneficial molecular subroutines or pathways. We also envisage that in future it may emerge that in certain contexts or conditions, Hyp-PDT is superior to chemotherapeutics or targeted therapeutics in inducing ICD and anticancer immune responses.

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Figure Legends:

Figure 1. Contribution of Hypericin-based Photodynamic Therapy (Hyp-PDT) and chemotherapy towards the advancement of the concept of immunogenic cell death (ICD). CRT – calreticulin; DAMPs – damage-associated molecular patterns; Ecto – surface exposed/tethered; ER – endoplasmic reticulum; HMGB1 – high-mobility group box 1; ROS- reactive oxygen species;

Figure 2. Hypericin-based photodynamic therapy (Hyp-PDT) is evolutionarily destined to be associated with danger signalling? A schematic representation of how hypericin helps the plants that produce it e.g. *Hypericum perforatum* perform *de novo* PDT to kill pests and how this signalling pathway is partly controlled by plant cell-derived damage-associated molecular patterns (DAMPs) and jasmonic acid, a danger signalling elicitor in plants. Please see the text for more details.

Figure 3. A schematic representation of immunogenic cell death (ICD) induced by hypericin-based photodynamic therapy (Hyp-PDT). Hyp-PDT causes cancer cells to emit spatiotemporally defined combination of danger signals which causes formation of fully mature immunogenic DCs, clonal proliferation and activation of T lymphocytes and potent instigation of tumour rejecting anti-cancer immunity *in vivo*.

Figure 4. Autophagy ablation accentuates Hyp-PDT induced ER stress. A375m human melanoma cells (A) and T24 human bladder cancer cells (B) were either used as mock transfected wild-type cells (CO or SCR) or transfected with shRNA (A) or siRNA (B) directed against ATG5 causing $ATG5^{KD}$. These respective cells were then either left untreated (CNTR) or treated with a relevant dose of Hyp-PDT (150 nM hypericin incubation for 16 h in serum-containing media followed by irradiation with 2.16 J/cm^2 fluence dose). Following this, the cells were recovered at the indicated time-points; their lysates were created and were immunoblotted with antibodies against the mentioned proteins relevant for ER stress analysis. A densitometric analysis is shown for the levels of phosphorylation of eIF2α protein for A375m cells (C) and T24 cells (D).

Table 1 - Glossary:

Abscopal effects: A phenomenon where local treatment of tumours causes shrinking of not only the treated areas but also the distant non-treated ones (e.g. metastasized cancer cells). Mainly applies to non-drug based therapies like radiotherapy and PDT.

Acidosis: Increased acidity (pH < 7.35) of the tumour microenvironment.

Anti-tumorigenic immune cells: Immune cells that actively take part in direct or indirect targeting and elimination of tumour cells e.g. cytotoxic T cells, NK cells and Th1 lymphocytes.

Chaperokine: Chaperones capable of exhibiting cytokinetic functions like inducing immune cell chemotaxis and immune cell differentiation.

Clinically-relevant tumour: A tumour visible to clinical diagnostic analysis and macroscopic or probe-based imaging. Of note, most neoplastic lesions are not detectable at their earliest stages of progression.

Curative syngeneic murine model: A murine model where tumour transplantation precedes immunization with a vaccine or dying/dead cancer cells.

Danger signalling: A signalling cascade activated in cancer cells for trafficking of danger signals or DAMPs towards the extracellular environment.

Drug-efflux channels: Molecular channels that actively bind and remove a drug from a cell's cytoplasm.

Dysfunctional immune microenvironment: A tumour environment where the immune cells are incapable of effectively targeting and eliminating cancer cells.

Endokine: A cytokine molecule that is retained within the cells (mostly in the nucleus) and performs non-immunological function in healthy cells.

Fully mature immunogenic DCs: DCs that not only exhibit phenotypic and functional maturation but also exhibit very low levels or total absence of immunosuppressive cytokines like IL10 and TGF-β.

Immunosurvelliance: A monitoring process of the immune system that entails detection and elimination of cancer cells.

Mitochondria-associated membranes (MAMs): MAMs are membranous structures playing the role of physically tethering the ER and the mitochondria.

Mitophagy: A macroautophagic process aimed at predominantly eliminating damaged mitochondria.

Opsonisation: A process entailing marking a target for ingestion and destruction by phagocytes.

PDT paradigm: In this context 'paradigm' refers to a set of results that are applicable to a specific photosensitiser, under conditions of light-activation. Thus, this term does not refer to general PDT features but rather describes biologically relevant characteristics of a type of photosensitiser, e.g. phox-ER stress based ICD, is specific to the PDT paradigm utilizing hypericin.

Physiological apoptosis: Programmed death-routine that enables silent immunoclearance of the dying cell.

Pro-inflammatory transcription factors (TFs): TFs that produce transcripts that code for proteins functioning during inflammation e.g. NF-kB.

Prophylactic syngeneic murine model: A model where immunization with a vaccine or dying/dead cancer cells precedes tumour transplantation.

Pro-tumorigenic immune cells: Immune cells that encourage survival and proliferation of tumour cells e.g. Treg cells and myeloid-derived suppressor cells.

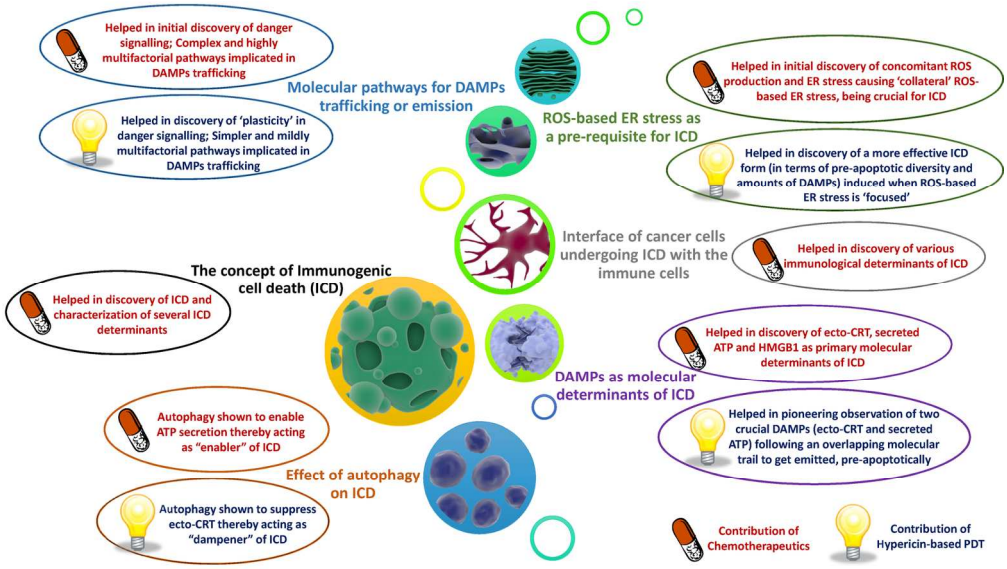
Respiratory burst: Rapid release of reactive oxygen species from immune cells aimed at eliminating the target via severe oxidative stress.

Reticulophagy: A macroautophagic process aimed at predominantly eliminating damaged reticular structures like the ER.

Tolerogenic cell death: A cell death-routine that enables silent immunoclearance of the dying cell.

Tolerogenicity: The act of spreading tolerance towards a set of antigens possessed by a target cell.

Tumour-promoting cytokines: Cytokines that encourage survival and proliferation of tumour cells by acting as *de facto* growth factors.



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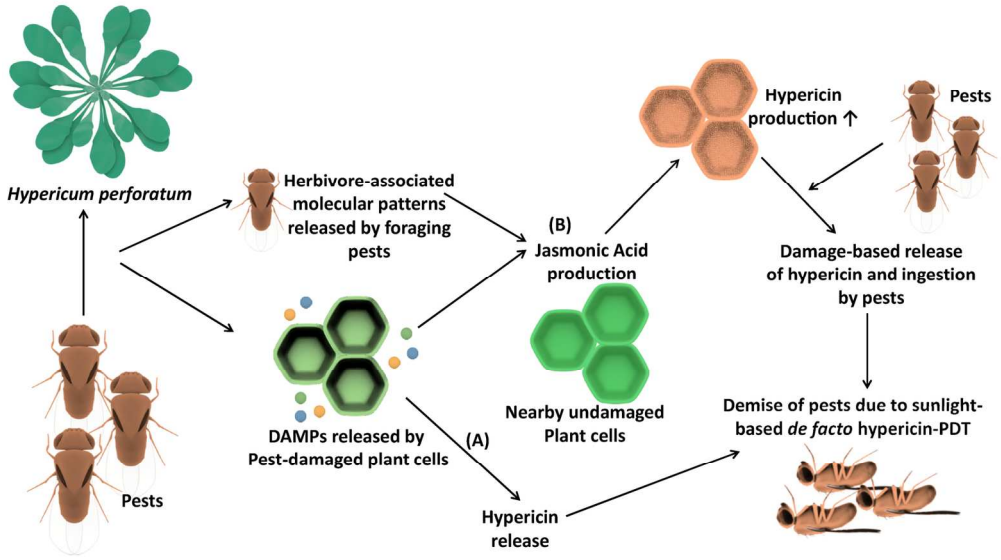

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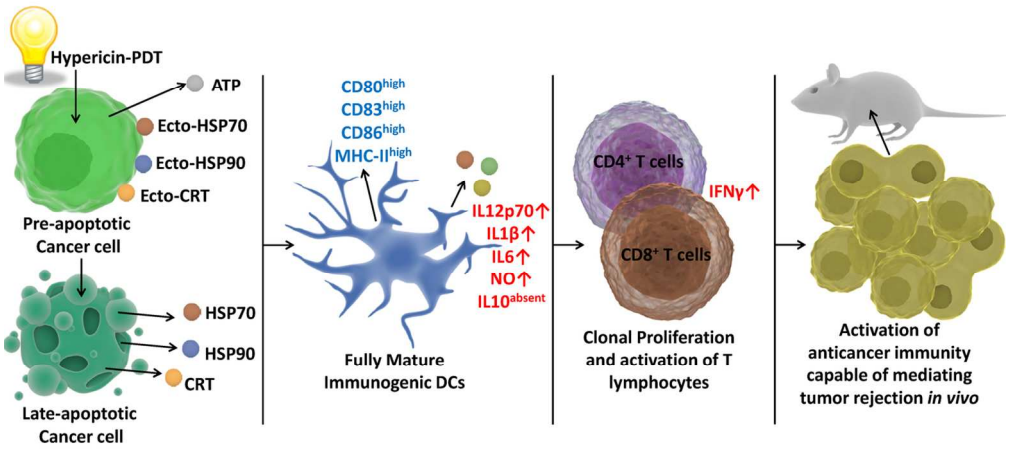

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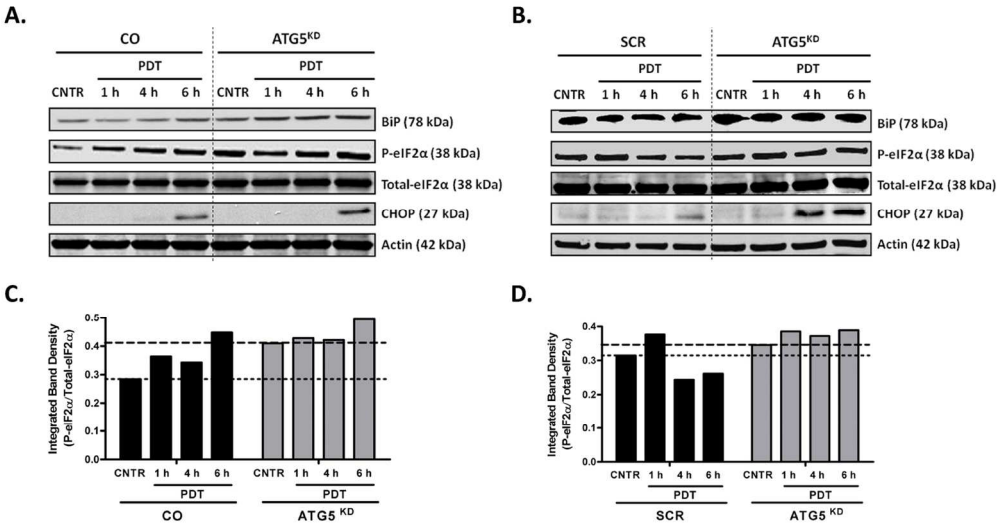

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