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Use of anti-cancer drugs, mitocans, to enhance the immune responses against tumors

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Abstract

Cytotoxic drugs in cancer therapy are used with the expectation of selectively killing and thereby eliminating the offending cancer cells. If they should die in an appropriate manner, the cells can also release danger signals that promote an immune reaction that reinforces the response against the cancer. The identity of these immune-enhancing danger signals, how they work extra- and intracellularly, and the molecular mechanisms by which some anti-cancer drugs induce cell death to bring about the release of danger signals are the major focus of this review. A specific group of mitocans, the vitamin E analogs that act by targeting mitochondria to drive ROS production and also promote a more immunogenic means of cancer cell death exemplify such anti-cancer drugs. The role of reactive oxygen species (ROS) production and the events leading to the activation of the inflammasome and pro-inflammatory mediators induced by dying cancer cell mitochondria are discussed along with the evidence for their contribution to promoting immune responses against cancer. Current knowledge of how the danger signals interact with immune cells to boost the anti-tumor response is also evaluated.

Keywords

Mitocans; immunotherapy; inflammasome; cancer therapy; reactive oxygen species; mitochondria

Immunogenic Cancer Cell Death

Cancer cells can undergo several alternative modes of cell death depending upon the cytotoxic drug treatment applied and the conditions in the tumor microenvironment. However, certain forms of drug-induced cell death are much more effective than others because of their ability to activate potent anti-cancer immune responses. It is well known that infectious pathogen-associated molecular patterns (PAMPs) activate immune cells through pattern recognition receptors (PRRs). Similarly, cellular injury can release endogenous 'damage'-associated molecular patterns (DAMPs) or alarmins that activate immunity (reviewed in [1]). Therefore, it is important to understand at the molecular level the cytotoxic events induced in cancer cells by chemotherapy and the DAMP signals that these dying cells are capable of releasing. It should then become possible to selectively apply anti-cancer drugs with the aim of provoking the type of cell death induced that will promote a potent immune reaction to help eliminate the cancer. Over the past several years, evidence has been mounting that the DAMPS released by dying cancer cells has a strong bearing on the ability of the host to develop a suitable immune response that will help to eliminate remaining live cancer cells and provide sustained immunity. Factors that impact on this outcome are the focus of this review.

Mechanisms of Cancer Cell Death and Their Relevance To Anti-Cancer Immune Responses

Apoptosis was originally believed to represent a “tidy” form of cell death whereby intracellular components were constrained within membrane encased cellular fragments which were then engulfed and cleanly removed by macrophages thereby avoiding the potential for autoimmune problems. However, the recent discovery of the inflammasome response (detailed below) has significantly improved the understanding and development of protective immune responses targeting cancers and reawakened studies on the role of cytotoxic drug-induced cell death in inflammation. Thus, intracellular mediators released from dying cancer cells provide essential signals that promote innate immune responses and facilitate immune cell recognition of tumor cells and generation of protective adaptive immune responses.

The different types of cell death

Numerous reviews have recently been published distinguishing between the properties and characteristics of the various forms of cell death [2-6] and they are only briefly discussed here. Historically, apoptosis and necrosis represented two extreme forms of a broad spectrum of cell death modes. In this review, the four main cell death programs of autophagy, necrosis, pyroptosis and apoptosis are described as alternative pathways and more importantly, examined in the context of their impact and relevance to the immune system. In 1988, the discoverers of apoptosis reviewed the differences in what were then identified as the two predominant and distinct forms of cell death, necrosis and apoptosis [7], that have since become well recognised and characterised molecularly. The following is a quotation from this report: “Necrosis is a degenerative phenomenon that follows irreversible injury. Apoptosis, in contrast, appears to be an active process requiring protein synthesis for its execution... Morphologically, apoptosis involves condensation of the nuclear chromatin and cytoplasm, fragmentation of the nucleus, and budding of the whole cell to produce membrane-bounded bodies in which organelles are initially intact. These bodies are disposed of by adjacent cells without inflammation. Biochemically, there is distinctive internucleosome cleavage of DNA in apoptosis, which is quite different from the random DNA degradation observed in necrosis.”

Already it can be seen from the last sentence that the authors were well aware of the fact that DNA degradation was a common factor in cell death, albeit that differences in the nature of this degradation were apparent. Apoptosis has since become molecularly characterized as programmed cell death involving a sequence of orchestrated events mediated by either an extrinsic (external) or intrinsic (internal) activation pathway leading to a characteristic type of nuclear DNA fragmentation. The intrinsic pathway of apoptosis involves the mitochondria whereby several pro-apoptotic proteins including cytochrome c are released from the intermembraneous space in mitochondria following formation of a mitochondrial outer membrane permeability pore or MOMP. When cytochrome c is released through the MOMP channel it is recruited into a large protein complex known as the apoptosome – a scaffold platform that binds to other proteins in the cytosol including Apaf-1 and the protease precursor, pro-caspase-9. The apoptosome cleaves pro-caspase 9 to the proteolytically active form caspase-9, which in turn activates the effector protease, caspase-3, triggering the execution phase of apoptosis. At this point, the cytoplasmic DNase (CAD) is activated and this enzyme leaves the cytosol, enters the nucleus, and induces DNA fragmentation by cleaving internucleosomal DNA, causing chromatin (DNA and histones inside the nucleus) to condense with other proteins. The dying cell under the influence of the proteolytic action of caspases also undergoes phosphatidylserine externalization causing the cell membrane to bleb, forming vesicles that contain condensed former components of the

cell which fragment into several apoptotic bodies that are phagocytosed by macrophages or adjacent cells.

In contrast to apoptotic events, necrotic cells result from the rapid loss of cell homeostasis as intracellular ATP stores become depleted. The necrosing cells swell (oncosis) as water and ion influx occurs increasing their volume, leading to rupture and associated loss of cellular organelles and cell membrane integrity. Apoptotic bodies or blebs do not occur and the nuclear chromatin in necrotic cells is irregularly clumped but without extensive changes in nuclear condensation. Leakage results in release of cellular contents which can promote inflammation.

Two other forms of cell death have been identified that are in between necrosis and apoptosis and are described as autophagy and pyroptosis. They also show opposing outcomes on the immune response. Pyroptosis is similar to necrosis in that the dying cells also lose cell membrane integrity, resulting in an even more highly inflammatory process. The name pyroptosis refers to the fever induced by the inflammatory response associated with this form of cell death with plasma membrane rupture. The nuclear modifications occurring during pyroptosis show greater similarities to the type of DNA fragmentation and nuclear condensation detected in apoptosis except that a caspase-1 activated DNase (identity unknown) produces a more necrosis-like smeared DNA cleavage without nuclear fragmentation which differs from the distinct form of oligonucleosomal fragmentation pattern and laddering with nuclear fragmentation that is the hallmark of apoptosis [8]. Apoptotic cells fragment into discrete apoptotic bodies unlike pyroptotic cells, which do not. Another characteristic feature of pyroptotic cells is the activation of caspase-1 to cleave the precursor forms of the cytokines pro-IL-1 β and -IL-18 resulting in the secretion of their mature active forms promoting inflammation.

Similar to apoptosis, autophagy is believed to be a more immunologically silent process by which cells can undergo death and autophagic cells maintain their membrane integrity. However, they exhibit a profound vacuolization as a result of organelle fusion and enveloping membrane-based autophagosome formation.

Contrary to the earlier popular belief about a tidy apoptosis induced by chemotherapy, apoptosis has since been shown to induce potent immune reactivity under the right circumstances. Hence, in order for strong immune responses to be induced during the cell death process, including by apoptosis, activation of inflammasomes is required resulting in the caspase mediated proteolytic maturation of the pro-inflammatory cytokines, interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) from their respective precursor molecules and subsequent release. This pro-inflammatory response occurs either as a result of dying cells releasing these signals or indirectly by the release of danger signals alerting and activating nearby immune cells. In an effective response, IL-1 β release will ultimately promote the maturation and activation of CD8 $^{+}$ CTL immune responses essential for the longer term killing and elimination of tumor cells [9][10]. A vast range of extra- and intracellular generated danger signals are released by dying cancer cells and some of these relevant to this review are described in the ensuing sections. Particularly the essential mediators signalling and activating the inflammasome response in bystander immune cells such as dendritic cells (DCs) to promote the adaptive immune responses are discussed in detail.

Structure and Function of Inflammasomes

One of the greatest advances in understanding the immune system over the past decade has been the discovery of inflammasomes (reviewed in [10-12]). As the name suggests, inflammasomes are responsible for activating inflammatory processes, [11] and have been

shown to induce cell pyroptosis, a process of programmed cell death distinct from apoptosis [3, 8]. These intracellular pro-inflammatory alarm signalers and cytosolic sensors respond to a variety of danger signals including toxic substances or component materials derived from infectious agents such as viruses, bacterial pathogens or other components of dying cells. Hence, the particular inflammasome activated will depend on the type of danger signal binding to and assembling the complex, providing its specificity. From this large family of cytosolic multiprotein complexes, three of the more characterized forms relevant to this review; NLRP3, AIM2, and IPAF, will be described as representative examples (Fig. 1). The complete assembled inflammasome structure has not yet been determined but likely resembles that of the Apaf-1/apoptosome and the related wheel of death, the CD95/Fas death-inducing signaling complex (DISC), involved in apoptosis [13, 14]. Thus, inflammasomes also contain different caspase enzymes (aspartic acid targeting, cysteine-dependent proteases) comprising part of their activity. A hallmark of inflammasome activation is that they promote the maturation of the pro-inflammatory cytokines, IL-1 β and IL-18.

Although caspases -3, -8 and -9 are recognised for their essential roles as pro-apoptotic mediators of cell death, the other members of the caspase family, particularly caspase-1 (aka ICE), are pro-inflammatory because they are important in regulating secretion of the cytokines IL-1 β and IL-18, cleaving the precursor or immature forms of these cytokines to facilitate release of the mature, active factors. The pro-inflammatory caspases include caspase-1, caspase-4 and caspase-5 in humans and caspase-1, caspase-11, and caspase-12 in the mouse.

Inflammasomes are large molecular complexes of proteins forming recruitment platforms to activate the inflammatory caspases in a similar way that the apoptotic caspases -8 and -9 are activated by the DISC and the Apaf-1/apoptosome, respectively [13, 14]. The inflammasome complexes include nucleotide-binding oligomerization domain (NOD1/Apaf-1 family, NACHT or NOD-like) receptor (NLR) family proteins (Fig. 1). These proteins, also known as the caspase recruitment domain (CARD) family play a very important role in the immune system. They belong to the superfamily of intracellular pattern/pathogen recognition receptors (PRRs) that are conserved in evolution from plants to animals and recognize molecules containing specific structural components derived from pathogens such as viruses and bacteria [9-12]. The C-terminal portions of these proteins contain leucine-rich repeat (LRR) domains involved in ligand binding/recognition interactions. The middle of the protein is characterized by a NOD involved in protein self-oligomerization. The N-terminal portion contains the CARD regions required for the caspase binding and activation that then leads to apoptosis and cytokine expression pathways [15, 16]. Mutations in the NLR genes that encode these proteins have often been associated with genetically linked inflammatory diseases, once again indicating the important roles they play in the immune response.

Danger Signals

ATP release during cell death as an important danger signal activating inflammasomes

Many different danger signals have been found to activate inflammasomes (see reviews [17, 18]) and one of the most characterised and directly relevant to this review is cellular ATP, as an important energy source derived from glycolysis and also supplied by mitochondria. Tumor cells contain millimolar levels of ATP and apoptosis releases much higher levels of cellular ATP (~100 nM, [19]) compared to necrosis, where ATP levels in the dying cells are greatly depleted during cell death. In studies investigating the ability of a range of different cytotoxic agents including some chemotherapeutic drugs (cadmium, etoposide, mitomycin C, oxaliplatin, *cis*-platin, staurosporine, thapsigargin, mitoxanthrone, doxorubicin) to induce

ATP release from cancer cells, it was found that intracellular ATP concentrations decreased before and during early apoptotic events such as dissipation of the mitochondrial transmembrane potential or exposure of phosphatidylserine on the plasma membrane [19]. Then, as apoptosis progressed, intracellular ATP levels further declined and ATP was released extracellularly by an undefined mechanism, and even advanced-stage apoptotic cells were shown to still contain sizeable residual ATP. When cellular ATP levels became extensively depleted, the cells then entered into necrosis as a secondary response (secondary necrosis) [19]. Many studies have revealed that it is possible to switch apoptotic cell death to necrosis by reducing cellular ATP levels upon inhibiting mitochondrial energy production [20-23]. Hence, ATP release is characteristic of apoptotic cell death induced by conventional anti-cancer therapies, but is unlikely to result from necrosis or autophagic processes in which ATP becomes depleted.

More recent studies showed that the ATP released by apoptosing cells is important for tumor cell immunogenicity. Thus, oxaliplatin or anthracycline treated colon cancer cells admixed with ATP scavengers (antimycin A plus glucose or apyrase or dinitrophenol) or treated with inhibitors of ATP binding to purinergic receptors, abolished the capacity of the tumor cells to elicit protective antitumor immune responses upon subcutaneous inoculation of the cells into normal mice (reviewed in [24]). Together with the observed general release of ATP by multiple distinct cell death inducers, this led to the proposal that dying tumor cell ATP release is indispensable for their immunogenicity [24]. Earlier studies had revealed that extracellular ATP release at micromolar levels would be sufficient to activate and promote migration of macrophages producing IL-6 [25, 26]. In more elaborate studies with apoptotic tumor cell supernatants obtained using apoptosis induction (either by UV- induced DNA damage, FAS receptor-mediated or dexamethasone/steroid-induced), 100 nM extracellular ATP release by chemotherapeutic agents was shown to be sufficient to act as a “find-me” signal and promoted chemotactic attractant binding via the purinergic receptor P2Rx₇ on the surface of phagocytes such as monocytes, macrophages and dendritic cells (DCs), leading to their recruitment and the subsequent disposal of the debris from the decomposing cells [27]. Hence, the role of sub-micromolar cellular ATP release as an immunogenic danger signal is well supported by the experimental data.

Cytosolic DNA damage sensing systems for double stranded (ds) DNA are key internal triggers of pro-inflammatory responses including inflammasome activation in dying cancer cells

Although many of the cell death pathways include nuclear DNA fragmentation as an outcome as described in section 1.2 above, the role that these dsDNA fragments and associated DNA bound proteins play in the direct intracellular activation of the inflammasomes by anti-cancer drugs has not yet been well characterized. Nevertheless, in situations that have been studied in which viral or microbial infection or tissue damage leads to the presence of cytosolic DNA, this is quickly followed by the potent activation of the innate and adaptive immune responses. For this to occur, a range of different cytosolic DNA sensors become activated with ensuing induction of type-I interferons (IFNs), pro-inflammatory cytokines and chemokines, characteristic of innate immune responses (reviewed in [28]). Not surprisingly these cytosolic dsDNA sensing systems show a high level of redundancy such that if one is counteracted or lost, then other alternatives will operate.

Historically, the earliest described cytosolic DNA sensors were DAI [the DNA-dependent activator of the interferon regulatory transcription factors (interferon regulatory factors or IRFs)] [29, 30], and absent in melanoma 2 (AIM2) [29, 31-33], the latter being an inflammasome that triggers the innate and adaptive immune systems. DAI (also known as DLM-1/ZBP1) is different from the inflammasomes as it does not contain a CARD, but

functions rather to induce expression of IFNs by triggering the TANK binding kinase (TBK)-1/interferon regulatory factor (IRF)-3-dependent type I IFN response [34] (Fig. 2). DAI contains three DNA binding domains in its N-terminal region, Z₁, Z₂ and D3, with the D3 region critical for DNA binding and oligomerization. DAI binds to poly(dA-dT) (B-DNA). In the C-terminal region are located three receptor-interacting protein (RIP) homotypic interaction motifs (RHIMs) and when arrayed along the length of DNA, these domains provide scaffolding for recruiting the RHIM-containing kinases, RIP1 and RIP3 which signal by phosphorylation and activation of NF-kappaB and IRF-3 transcription factors to induce type I IFN gene expression [35, 36]. Thus, knockdown of RIP1 or RIP3 was shown to greatly reduce the DAI-induced NF-kappaB activation.

Other cytosolic DNA sensors that have been identified to date include the RIG-I-like receptors (RLRs), retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5), although these are better known as sensors for RNA as they act indirectly by facilitating the induction from cytoplasmic DNA of an RNA polymerase III-transcribed RNA intermediate [37-40].

Although the role of DNA fragments in anti-cancer drug-induced cell death and ensuing inflammatory responses has not yet been extensively characterized, it is possible to examine the effects that anti-cancer drugs such as doxorubicin exert on cancer cells and obtain some insight into the roles of cytosolic DNA fragments in this process. Hence, from as early as 1994, doxorubicin treatment of whole rat glomeruli and dissociated mesangial and resident glomerular macrophage cells was found to induce secretion of IL-1beta [41], consistent with inflammasome activation. This observation also provided an explanation for the drug induced inflammatory nephrosis that was associated with doxorubicin treatment. Subsequently, analysis of rat cardiomyocytes after *in vivo* treatment with doxorubicin showed increased oxidative stress and mitochondria-mediated apoptosis as a result of cytochrome c release, associated in the longer term with adaptive protective responses of increased oxidative phosphorylation, ROS production, superoxide dismutase activity, and BCL-2:BAX ratio [42]. Comparative analyses between normal cells (such as glomeruli tissue and cardiomyocytes) and tumor cells to doxorubicin-induced inflammasome activation may help to understand the different responses of both cell types to such chemotherapeutic drugs.

Importantly, the extent of apoptosis was measurable by the amount of *cytosolic* mononucleosomal and oligonucleosomal DNA fragments (180 bp or multiples), which was significantly increased by drug treatment. Subsequently, doxorubicin-induced apoptosis of MCF-7 breast cancer cells was shown to involve activation of caspase-1 and the IPAF inflammasome and caspase 1-dependent apoptosis induced by doxorubicin was inhibited by BCL-2. In addition, mitochondrial membrane permeabilization induced by caspase-1 and activated IPAF resulted in the activation of BAX in mitochondria [43]. Thus, although it is not clear exactly how the anti-cancer drug doxorubicin activated inflammasomes, based on the above results it is highly likely that cytosolic DNA fragments acting in combination with mitochondrial ROS are important signals in the processes leading to the activation of the IPAF/caspase-1 inflammasome response.

Pro-inflammatory responses to cytoplasmic DNA fragments produced during retroviral, DNA viral or bacterial infections are likely to arise during cell death events associated with infection as well as from cytotoxic chemotherapeutic drug treatment. One of the best examples of the effects of cytosolic DNA fragments comes from studies where poly(dA:dT) transfected into the cytosol of bone marrow derived macrophages (BMM) or human THP-1 acute monocytic leukemia cells activated the ASC/AIM2 inflammasome response and resulting pyroptosis [31, 32, 44, 45]. In this manner, cytoplasmic DNA also caused

upregulation of MHC Class I, induction of IFN and other cytokines as well as cell death [45]. The observed toxicity was a specific response to dsDNA and not ssDNA and depended on the length of transfected DNA with very short ds oligonucleotides unable to efficiently induce cell death, whereas 44–base pair (bp) DNA transfected at high concentration efficiently killed the cells [45]. These events, including both cytokine induction and cell death, were shown to be independent of recognition of “CpG motifs”, ruling out a role of the TLR9 receptor. Rather, they indicated a more direct, intracellular activation process was occurring depending on cytosolic dsDNA fragment length, with 100bp fragments even more effective [45]. In support of this, knockdown studies revealed the DNA binding HIN-200 family protein, p202, to be a regulatory protein that inhibited the dsDNA-induced caspase-1 and -3 activation. Conversely, the related pyrin domain-containing HIN-200 related factor, AIM2 (p210) was required for caspase-1 and -3 activation by cytoplasmically introduced dsDNA [45].

AIM2 is a non-NLR family member (in that it has no NOD domain) but acts as an inflammasome scaffold, and oligomerization of this complex involves clustering by multiple binding sites across its ligand, dsDNA, to which AIM2 binds via its C-terminal HIN domain [31, 32, 45, 46] (Fig. 1). AIM2 multimerises along the length of cytosolic DNA fragments providing a scaffold leading to the formation of the AIM2/ASC inflammasome complex (reviewed in [10]). AIM2 contains a region that shows homology to a domain in the protein, PYRIN, originally discovered in relation to the genetic autoimmune disorder, familial Mediterranean fever (FMF) and hence was given the name PYRIN to indicate fever. The PYRIN domain (PYD) in AIM2 recruits the adaptor protein, apoptosis-associated speck-like protein (ASC), through homotypic PYD interactions to build the AIM2/ASC inflammasome complex (Fig. 1). ASC then recruits caspase-1, leading to proteolytic caspase auto-activation and production of IL-1 β and IL-18 [45-47]. AIM2, like DAI, is a cytosolic double-stranded DNA (dsDNA) sensor, but unlike DAI which induces type I IFNs, AIM2 is distinct in that it induces caspase-1-dependent IL-1 β and IL-18 maturation [31, 32, 45-47].

AIM2 and NLRP3 both contain a PYD that interacts with ASC via homotypic PYD-PYD interactions enabling the ASC CARD domain to then recruit pro-caspase-1 to the growing assemblage of the inflammasome complex. As for other inflammasomes, upon autoactivation, caspase-1 directs pro-inflammatory cytokine maturation and secretion (such as IL-1 β and IL-18). Ligands including any cytosolic dsDNA from viruses, bacteria, or the host cell nucleus itself will activate the AIM2 inflammasome [31, 32, 45-47]. More studies are required to determine the significance and relative importance of the AIM2 pathway in anti-cancer drug-induced cell death pathways of inflammasome activation.

HMGB DNA Binding Proteins

Another type of sensor for DNA associated danger signals is the high mobility group box (HMGB) family of DNA binding nucleoproteins 1, 2 and 3 ([48] reviewed in [49]). HMGB1 released from dying cells after DNA fragmentation binds to TOLL receptors (TLRs), thereby activating DC cells to induce IL-1 β production and secretion. HMGB1 can be released from either necrotic or apoptotic cells, although during apoptosis it is in an oxidised state that is inactive as an inflammatory molecule. However, when cells die via autophagy leading to necrosis, HMGB1 is released in a non-oxidised state as a powerful pro-inflammatory signal. A large range of partner molecules bind HMGB1 including cytokines, endotoxins, nucleosomes and DNA and this results in different interactions of HMGB1 complexes with cell surface receptors, including the receptor for advanced glycation end products (RAGE) and it promotes pro-inflammatory signals including activation of transcription factors, IRF-3 and NF-kappaB.

Collectively, it is clear from the above studies that the DNA damage initiated during cytotoxic drug-induced death of cancer cells can activate the inflammasome responses by several different mechanisms, both intra- and extracellularly mediated.

Cellular ROS production, inflammasome activation and release of IL-1 beta

The role of ROS in activating the inflammasome is supported by studies using a variety of different inflammasome signalling modulators, ROS inhibitors, or pro-oxidants like H₂O₂ and/or anti-oxidants (reviewed in [50, 51]). Significantly, the evidence indicates that for inflammasome activation, ROS can emanate from different cellular sources including mitochondria or cytosolic NADPH oxidases. For example, Tschopp's group [52] showed that activation of the NLRP3 inflammasome by asbestos or silica was dependent on ROS generated by an NADPH oxidase and mitochondria did not appear to be required because they could be inhibited with respiratory complex inhibitors rotenone or TTFA without affecting subsequent IL-1beta release from the human acute monocytic leukemia cell line, THP-1. However, this result is inconclusive since two independent studies with primary monocytes derived from patients with chronic granulomatous disease (CGD) containing mutant, defective cytosolic NADPH oxidases revealed no differences in the secretion of IL-1beta induced by the NLRP3 inflammasome activators uric acid, silica, imiquimod or LPS [53, 54]. In addition, earlier studies of CGD patients with defective NADPH oxidases showed no impaired production of superoxide anion or H₂O₂ upon stimulation of their polymorphonuclear cells or skin fibroblasts by TNF or IL-1 [55]. Hence, alternative sources such as mitochondrial ROS production probably occur and still explain inflammasome activation.

Role of mitochondria in ROS production activating inflammasomes

Two examples of specific inflammasome activating signals for which the role of mitochondria and ROS has been confirmed are silica [56] and ATP [57], both resulting in activation of NLRP3 inflammasome and release of pro-inflammatory cytokines [58] in a process that involves ROS. Thus, in a careful study on the role of mitochondria in silica-induced apoptosis of primary alveolar macrophages, pre-treating cells with the mitochondrial targeting rhodamine 6G at levels that inhibited ATP and mitochondrial ROS production also inhibited cytokine release [56]. However, treating the cells with exogenous H₂O₂ [0.1mM] or the superoxide inducing pyrogallol overcame this inhibition. In this regard, H₂O₂ and antioxidants like N-acetyl-L-cysteine have long been known to have opposing effects on the activation of NF-kappaB leading to IL-1beta and TNFalpha cytokine release [59, 60]. It is also noteworthy that the release of these cytokines enhances ROS production, and hence, can feed back in a self-stimulatory manner [61, 62], thereby amplifying the response.

The second example which points to an essential role for mitochondria as a major source of ROS comes from studies of the intracellular effect of ATP as a danger signal as described in section 1.2.3 above. Extracellular ATP is known to bind via purinergic receptors (P2×7) causing a rapid pulse of ROS production in alveolar macrophages within minutes of addition and leading to downstream PI3K/Akt and ERK1/2 activation [57, 63]. ROS was shown to act via the glutathionylation and inhibition of PTEN phosphatase, thereby favouring PI3K activation leading to activation of caspase-1. However, caspase-1 activation by ATP was inhibited when the general flavoprotein inhibitor, diphenylene iodonium (DPI), that is proposed to directly inhibit cytosolic NADPH oxidases, was used to block NADPH oxidases. In addition, in agreement with a previous report [64], release of pro-inflammatory cytokines (IL-1beta and IL-18) did not occur upon ATP stimulation via the P2×7 receptor alone, but also required pre-activation with the TLR4 ligand, LPS [50]. These results

suggest that ROS alone, although shown to activate caspase-1, requires in addition an NLR activator to enable complete inflammasome formation and activation.

In mammalian cells, mitochondria are a major source of ROS important for diverse events such as cellular proliferation, differentiation and migration (reviewed in [65, 66]). Understanding the role of ROS in cellular function has advanced to the stage where cellular “redox” signalling is accepted to be involved in regulating normal processes and disease progression, including angiogenesis, oxidative stress, aging, and cancer. Now it is possible to add inflammatory responses. A key point is that excessive ROS production from mitochondria overloads the thiol redox system and causes progressive inactivation of thiol containing anti-oxidant proteins such as the reducing enzymes, sulfiredoxins and thioredoxins, thereby compromising redox homeostasis. Thus, once mitochondrial ROS levels overload the threshold capacity of this anti-oxidant cellular system, the excessive ROS will continue to build up as increased superoxide (O_2^-) and H_2O_2 levels [67]. It is outside the scope of this review to describe in detail the production processes of mitochondrial ROS and the topic has been well covered in a recent review [68].

The role of oxidative stress in activating the inflammasome responses is further supported by many other studies where the ROS scavenger, N-acetyl-L-cysteine, has been used to negate ROS action [52, 54, 56, 57]. In addition, DPI, that is proposed to directly inhibit cytosolic NADPH oxidases, was found to greatly inhibit caspase-1 activation and reduce levels of secreted IL-1beta [54, 56, 57]. However, this effect of DPI was recently suggested to result from inhibition of IL-1beta gene expression rather than NADPH oxidase inhibition per se [64].

More recently, several specific and distinct mechanisms involving mitochondria in inflammasome activation have been identified. One signalling pathway involves ROS acting on the thioredoxin (TRX)-interacting protein (TXNIP), an inhibitor of TRX, to release it from TRX, allowing TXNIP to then bind and activate the NLRP3 inflammasome [69]. A second involves activation of one of the Nod-like receptor family – NLRX1, by signals including dsRNA, Shigella or TNFalpha [70]. The NLRX1 protein is mitochondrially localised and contains an N-terminal mitochondrial targeting sequence although its exact function has not been completely defined [71, 72]. One report showed NLRX1 interacted by binding via its CARD to that on the mitochondrial antiviral signalling protein, MAVS (also known as IPS-1, VISA or Cardif). The net effect of this binding was to inhibit MAVS function by sequestering and preventing it from interacting and activating the RIG-I or MDA-5 retinoic acid-inducible gene I (RIG-I)-like RNA helicase (RLH) receptors [72]. However, another study showed NLRX1 overexpression amplified ROS production, thereby mediating activation of the NF-kappaB and JNK pathways [70]. Further studies are required to determine the precise role of MAVS in the inflammasome activation pathway.

Biochemical studies of NLRX1 in HeLa cancer cells and HEK293T transformed cells showed that NLRX1 was imported into the mitochondrial matrix by a process involving proteolytic maturation and removal of the N-terminal mitochondrial targeting sequence and required the transmembrane potential [71]. In addition, NLRX1 was shown to co-immunoprecipitate with the UQCRC2 subunit of respiratory chain complex III. Given that complex III can be a key source of intracellular ROS production, it is tempting to speculate that NLRX1, upon maturation and import to the matrix face of mitochondria modulates complex III to activate ROS production, much like p66Shc does in *ras* transformed malignant cells [73]. More study will be required to resolve the precise nature of the relationships that exist between mitochondrial ROS and NLR function in activating inflammasomes.

The important collective message from the various results discussed above connecting mitochondrial ROS production activating inflammasomes and ROS scavengers inhibiting this process is that mitochondria are key intermediaries involved in inflammasome activation induced by a variety of different signals. This conclusion provides the basis for one of the key proposals raised in this review in that drugs targeting mitochondria to increase ROS production will promote an inflammasome response and lead to greater pro-inflammatory activation of immune cells, thereby increasing their anti-cancer effectiveness.

Ros Production and Activation of NF-Kappab and IRF-3 Promotes Pro-Inflammatory Cytokine Expression

Although the role of ROS released from mitochondria by targeted anti-cancer drugs in activating the NLRX1/MAVS pathway is not yet resolved, the indications are that ROS does activate this pathway. For example, when the classical inhibitor, rotenone was used as a mitochondrial complex I inhibitor, it was shown that in wild type cells, ROS produced by the dysfunctional mitochondria was predominantly responsible for enhanced RLR signaling and that antioxidants blocked the excess RLR signalling [74]. An essential role for MAVS in the innate immune response by RLR group RNA helicases RIG-I or MDA-5, which can be activated by dsDNA through the induction of an RNA polymerase III-transcribed RNA intermediate has been well established [37-40].

After MAVS activation, IRF-3, downstream of MAVS can bind to and activate cytosolic BAX, inducing BAX translocation to the mitochondria and initiation of the intrinsic apoptotic pathway. The RLR/MAVS signalling results in activation of transcription factors NF-kappaB and IRFs, including IRF-3 and IRF-7, which then induce pro-inflammatory cytokines and chemokines including IL-1beta, TNF, IL-6 and type I IFNs. MAVS is cleaved during apoptosis and when apoptosis is prevented by over-expressing the anti-apoptotic BCL-xL protein, MAVS cleavage is blocked placing this process downstream of caspase activation in the apoptotic program [75]. MAVS over-expression in cells induces caspase mediated apoptosis which is dependent on caspase localisation to the mitochondria in cells [76]. MAVS has also been shown recently to facilitate cell death by disrupting the mitochondrial membrane potential and by activating caspases [77].

Mitochondrial Targeted Anti-Cancer Drugs (Mitocans) Induce Apoptosis – Relationship To Ros Production and the Immune Response Against Cancer

The mitocan, vitamin E succinate (VES, alpha-tocopheryl succinate, alpha-TOS) selectively targets cancer cell mitochondria to induce cell death by triggering ROS production as one of the early events occurring within 30–60 min [78-80]. In particular, the pro-oxidant, α -TOS, has become well established as a potent inducer of ROS generation leading to apoptosis in cancer cells, but not in related normal cell types (reviewed in [71-73]). Alpha-TOS is preferentially selective for cancer cells because normal cells, particularly non-dividing cells, are much less sensitive to the apoptosis inducing effects of α -TOS. The lack of toxic effects on normal cells occurs because they are endowed with greater anti-oxidant defences and contain higher levels of esterase activity that inactivate the pro-oxidant α -TOS, releasing the succinate moiety (converting the pro-vitamin to the non-apoptogenic α -tocopherol, α -TOH). In general, malignant cells are more active than normal cells in producing O_2^- , are under intrinsic oxidative stress, and thus are more vulnerable to further damage by ROS-generating agents [81]. The intrinsic oxidative stress in cancer cells is also associated with increased SOD and catalase expression, presumably as a mechanism to tolerate increased ROS stress. These features make agents like α -TOS excellent candidates for cancer therapy, as it is eventually inactivated in normal cells and tissues and it works *in vivo*, suppressing

experimental malignancies in pre-clinical models such as colorectal or lung carcinomas, melanomas, mesotheliomas and breast cancers (reviewed in [82]).

The major form of ROS produced by cancer cells in response to -TOS is O_2^- because adding SOD removed the radicals and inhibited subsequent apoptosis [83, 84]. The site of O_2^- generation as well as the target of ROS action is the mitochondria because in studies where cancer cells were pre-treated with the mitochondrially targeted antioxidant, mitoquinone (Mito Q) [85], production of ROS was suppressed and induction of apoptosis inhibited following subsequent addition of -TOS was inhibited [80, 86]. The importance of ROS in mediating apoptosis induced by -TOS has been confirmed in several reports where the efficacy with which -TOS induced apoptosis closely paralleled ROS accumulation and cells with low anti-oxidant defences were more vulnerable to -TOS [83, 87]. More recently, -TOS was shown by biochemical analyses, genetic studies and to inhibit the succinate dehydrogenase (SDH) activity of the respiratory chain complex II by interacting with the proximal and distal ubiquinone (UbQ)-binding site (QP and QD, respectively) [88, 89]. Studies of immortalized Chinese hamster lung fibroblast cells with a dysfunctional mitochondrial respiratory chain complex II showed a failure of these cells to accumulate ROS or to undergo apoptosis in the presence of -TOS . Reconstituting the defective complex II in cells again restored sensitivity to -TOS . Similar resistance to -TOS was also observed when complex II subunits were knocked down with siRNA in wild type immortalized cells. These results indicated that -TOS displaced ubiquinone from binding to complex II, so that electrons arising from oxidation of succinate to fumarate were no longer accepted by their natural receptor and, instead, interacted with molecular oxygen to generate O_2^- [88].

In a follow up study [89], the Chinese hamster lung fibroblasts with functional, dysfunctional, and reconstituted complex II were transformed using H-RAS. The cells were then used to form xenografts in immunocompromised mice, and the results obtained from treating cells with -TOS either in culture or as tumors *in vivo* were again consistent with complex II as an essential mitochondrial target for the ability of the drug to kill cancer cells. The mechanism of killing is likely to involve O_2^- production which is then converted by SOD into highly diffusible H_2O_2 that activates the intrinsic apoptotic pathway leading to structural rearrangement and dimerization of cytosolic BAK or BAX via disulfide bridges and/or externalization of the BH3 C-terminal membrane-targeting sequence [88, 90-93]. The net result is to form channels in the mitochondrial outer membrane inducing apoptosis.

Cancer Immunotherapy and the Mitocan Class of Vitamin E Analogs

During the process of tumor development and tumor progression the immune system acts as a tumor suppressor poised to eliminate tumor cells by a process referred to as immune editing [94, 95]. This process in which both the innate and adaptive components of the immune system participate, involves continuous scanning and elimination of cancerous cells. However, progressively growing tumors can develop in the presence of a functioning immune system when tumor cells emerge that either display reduced immunogenicity or employ immunosuppressive mechanisms to weaken antitumor immune responses [96, 97].

The goal of the fast expanding field of tumor immunotherapy is to exploit the exquisite capacity of the immune system to recognize “non-self” to tip the scale in favor of the immune system towards recognition and elimination of the tumor. This objective can be achieved using a variety of approaches including infusion of *ex vivo* expanded tumor-specific cytotoxic T cells [98] or antigen-loaded DC [99, 100], administration of immunomodulatory agents which directly stimulate cells of the immune system [e.g. Toll-like receptor (TLR) agonists [101], cytokines (IL-2, IL-7, IL-15, IFN- γ , GM-CSF) [102], agonists of co-stimulatory molecules (e.g. anti-OX40 [CD134], anti-4-1BB [CD137]) [103,

104] or abrogation of regulatory mechanisms which dampen the immune response such as depletion of suppressive regulatory T cells (Treg) [105] or treatment with antagonistic (anti-CTLA-4, anti-PDL-1 anti-PD-1) antibodies [106, 107]. To varying degrees, these strategies have resulted in significant anti-tumor effects in several murine cancer models. However, these treatments have not translated into consistent objective clinical responses in humans [103, 108-110]. These observations have now led to the prevailing view that multi-modal therapy using a combination of agents that target different signaling pathways has a greater likelihood of therapeutic efficacy against established cancer.

Although chemotherapy as a frontline treatment modality of cancer is often effective in controlling tumor growth, it is plagued by undesirable toxicities such as marrow suppression and generalized immune suppression that are oftentimes life threatening. However, recent studies have revealed that at certain doses some conventional chemotherapeutic drugs behave as adjuvants that potentiate the anti-tumor immune response [111-114]. Evidence is mounting that some drugs induce immunogenic cell death characterized by specific cellular responses including up-regulation and/or release of endogenous damage associated molecular pattern (DAMP) molecules. For instance, anthracyclines such as doxorubicin result in the release of high mobility group box-1 (HMGB-1) proteins and translocation of calreticulin [111, 114, 115] and bortezomib causes heat shock protein (Hsp) up-regulation and release [116]. Furthermore, chemotherapeutic agents can modulate the immune response by directly acting on immune cells. A prime example is low dose cyclophosphamide therapy that preferentially depletes Foxp3-expressing regulatory T cells [113, 117] or inhibits their immune suppressive function [118].

Although there is a growing number of compounds that have recently been identified as mitocans (Table 1) and that have been shown to have anti-tumor effects [119], with few exceptions, little is known about their ability to modulate anti-tumor immune responses. Nevertheless, certain mitocans namely some of the thiol redox inhibitors, the electron transport chain targeting tamoxifen, the natural compound resveratrol, and in particular the ROS-modulating and Bcl-2 mimetic Vitamin E (α -tocopherol) analogs (VEA) have been examined for their immune modulating properties.

The thiol redox inhibitory isocyanates and arsenic trioxides have both been shown to increase natural killer (NK) and lymphokine activated killer (LAK) cell function [120, 121]. In particular, the isocyanate sulforaphane has been shown to increase NK cell activity and increase IL-2 and IFN- γ production of splenocytes [121] from Ehrlich ascite tumor bearing mice, suggesting an immune supportive role of this agent.

Among the electron transport chain targeting mitocans, tamoxifen has been one of the front-line therapies of estrogen-sensitive breast cancer, though studies examining the effect of tamoxifen treatment on the immune system revealed immune suppressive properties. While two studies report decreased NK cell activity in tamoxifen treated patients [122, 123], mitogen induced T cell proliferation was either inhibited [122] or increased [123]. Furthermore, *in vitro* studies of the direct effect of tamoxifen on human monocyte-derived dendritic cells (DC) showed that their ability to produce IL-12 and to induce T cell proliferation was diminished [124]. For these reasons, it has been suggested that tamoxifen may be better utilized in the treatment of immune-mediated disorders than in cancer immunotherapy [125].

The natural occurring resveratrol, found in grapes and grape products such as red wine, has recently garnered attention as an anti-tumor agent and a limited number of studies have examined the immunomodulatory properties of resveratrol in respect to tumor immunity. Using a murine colon cancer model, Yang et al. reported that resveratrol decreased the

number of immune suppressive regulatory CD4⁺CD25⁺ T cells and increased the number of INF- γ producing CD8⁺ T cells [124]. Increases in Treg cell numbers in tumor settings is often a major hurdle for successful immunotherapy [126]. However, this is in contrast to a study showing that resveratrol *in vitro* inhibited proliferation and IL-4 and IFN- γ secretion by T cells, and impaired B cell proliferation and antibody production [127]. In addition, resveratrol treatment of macrophages resulted in decreased co-stimulatory molecule expression making them poor T cell stimulators [127].

The inhibitory effects of resveratrol on antigen presenting cells was also corroborated in a recent study showing that resveratrol-treated DC had low expression of co-stimulatory molecules, exhibited low T cell stimulatory activity and did not secrete IL-12 upon activation [128]. Resveratrol's anti-tumor properties stem from its ability to induce apoptosis in tumor cells, but it has also been reported that resveratrol causes apoptotic death of activated T cells [129, 130]. The conflicting *in vivo* data of the beneficial decrease of Treg in tumor bearing mice and the apparent immune cell suppressive effects of resveratrol *in vitro* warrants more comprehensive research, but resveratrol may be a poor choice for tumor immunotherapy.

In this respect the Vitamin E (α -tocopherol) analogs (VEA), α -tocopheryl succinate (α -TOS) and α -tocopheryloxyacetic acid (α -TEA) have not only been examined for their anti-tumor activities in various rodent and human xenograft tumor models [131-138], but our research group has pioneered studies demonstrating the immune modulating properties of this novel class of anti-cancer drugs. The remainder of this review will focus on the current state of knowledge of how these VEAs interact with cells of the immune system to boost the anti-tumor response.

α -TOS and α -TEA as anti-cancer agents

α -TOS and α -TEA are semi-synthetic derivatives of vitamin E that structurally share the phytyl-tail and the chroman head with vitamin E (Fig. 3). While α -TOS differs from vitamin E in that the hydroxyl group at the number 6 carbon of the phenolic ring of the chroman head has been replaced by a succinic acid residue α -TEA has an acetic acid moiety at this position [135]. These drugs are highly hydrophobic in nature and are frequently delivered to cells or experimental animals in various oils [135], organic solvents (DMSO), ethanol [139, 140], or after vesiculation in phosphate buffered saline (PBS) [132, 138, 139]. In contrast to α -TOS that is susceptible to hydrolytic cleavage by intestinal esterases [131], the acetic acid moiety of α -TEA is attached via an ether bond (Fig. 3) [135], making it resistant to esterase hydrolysis and permitting stable delivery via the oral route. Oral gavage and aerosol delivery of α -TEA have been successfully employed to suppress primary tumor growth and reduce the incidence of metastasis in several murine tumor models [134, 135].

Hahn et al. recently reported, that when supplied to mice in the diet, α -TEA significantly inhibited the growth of a transplanted, highly metastatic breast cancer and dramatically reduced the incidence of lung metastases [132] and was able to delay the onset, and suppress the growth of spontaneous-arising breast cancer in transgenic MMTV-PyMT mice [133]. VEAs are particularly attractive as anti-tumor agents because of their selective toxicity towards tumor cells [137, 141-143] while displaying minimal toxicity towards immune cells [140]. Furthermore, *in vivo* administration of α -TOS did not interfere with TNF- α induced IL-12 production by dendritic cells or mitogen-induced proliferation of T lymphocytes (Fig. 4). Although the above referenced studies have established the efficacy of the VEAs against various forms of cancer, studies that have examined the mechanisms of VEA-induced anticancer activity have largely focused on the pro-apoptotic nature of these analogs [142, 144, 145], and we are only recently beginning to unravel the potential contribution of the immune system to the *in vivo* antitumor activity of these agents.

Combining Vitamin E Analogs as Anticancer Agents With Dendritic Cell Immunotherapy

Combination chemotherapy has emerged as a preferred modality of cancer treatment because of the potential to target multiple signaling pathways employed by tumor cells to escape drug or immune-mediated cell death. Unlike single drug therapy where the potential for host toxicity is high, combination chemotherapy allows for the use of lower drug concentrations with reduced toxicities to achieve clinical benefit. Recent data demonstrating that certain classes of chemotherapeutic drugs cause immunogenic tumor cell death which leads to enhancement of antigen cross-presentation and stimulation of the anti-tumor immune response have galvanized interest in chemotherapeutic agents as immune modulators [111-114, 146]. It is within reason to expect that combining immunotherapy with such immune modulating drugs will enhance the anti-tumor response while mitigating drug-related host toxicity. Our research group has pioneered the use of tumor-selective α -TOS and α -TEA mitocans as immunologic adjuvants to stimulate effective anti-tumor immune responses.

When administered in combination with *ex vivo* generated DC for the treatment of established murine Lewis lung and mammary cancers, α -TOS synergizes with DC to inhibit the growth of established primary tumors and suppress the formation of spontaneously-arising metastases [138, 140, 147]. The observed anti-tumor responses were strongly correlated with polarization toward a T helper 1 (T_H1) immune response evidenced by increased interferon-gamma (IFN- γ) secretion and enhanced tumoricidal activity by tumor-draining lymph node and splenic lymphocytes [138, 147]. An unexpected result from these studies was that non-antigen pulsed DC were as effective as TNF- α -matured DC in suppressing tumor growth [147]. In a similar fashion, α -TEA potentiated the efficacy of adoptively-transferred non-matured DC in reducing overall tumor burden and tumor multiplicity in a transgenic model of spontaneous murine breast cancer (Fig. 5). These intriguing findings led us to hypothesize that VEAs induce a form of immunogenic tumor cell death that results in the release of activating “danger signals” and putative tumor-associated antigens that can be ingested and efficiently cross-presented by DC to stimulate naïve antigen (tumor)-specific T lymphocytes. In support of this hypothesis, supernatant generated from α -TOS-treated tumor cells was shown to induce DC maturation evidenced by up-regulation of the co-stimulatory molecules, CD40, CD80 and CD86. The phenotypic change in DC maturation status was also accompanied by an increase in production of interleukin-12 (IL-12) [138, 147] that favors the development of a T_H1 immune response [148].

Although these studies indicated that VEAs transform immature DC to become more efficient stimulators of T cells capable of mediating tumor suppression, a pre-requisite for T cell activation by DC is efficient uptake, processing and loading of antigenic peptides onto MHC complexes for presentation. Regarding antigen uptake, we have shown that non-matured bone marrow-derived DC incubated with supernatants obtained from VEA-treated tumor cell were as efficient as TNF- α -matured DC in their endocytic and phagocytic functions (Fig. 6). Our experiments also demonstrated that components of the antigen-presenting machinery (APM) belonging to the multi-catalytic proteasome complex (delta) and the MHC class I loading complex (tapasin, ERp57) as well as surface expression of MHC class I itself were up-regulated in these DC (Fig. 7). The final essential step in DC-mediated T cell activation upon antigen acquisition, is migration to lymphatic organs for T cell priming. To this end, we have shown that VEA-treated tumor cell supernatant-pulsed DC demonstrate an increased ability to migrate *in vitro* toward CCL21, a chemokine that attracts antigen presenting and T cells to lymph nodes.

Vitamin E Analogs as Inducers of Immunogenic Tumor Cell Death

The mechanisms of α -TOS-mediated cell death has been thoroughly investigated [79, 142, 144, 149, 150]. Mitochondrial depolarization resulting in the generation of reactive oxygen species is considered an initiating event leading ultimately to apoptotic cell death [88, 151]. Similar to α -TOS, α -TEA stimulates mitochondrial depolarization and ROS generation (Fig. 8). A hallmark of immunogenic cell death is the up-regulation and/or release of DAMP molecules such as heat shock proteins and HMGB-1 proteins that play important roles in DC activation and enhancement of antigen cross-presentation [152-159]. As mentioned earlier, although this property has been described for a select group of chemotherapeutic drugs including anthracyclines [114] and taxanes [160], this property was recently identified with VEAs. Thus, tumor cells treated with α -TOS and α -TEA up-regulated expression and translocation of Hsp60, Hsp70 and Hsp90 to the cell surface [138, 147].

Supportive evidence for the role of Hsps released from tumor cells in response to VEA treatment as partly responsible for DC activation was shown by blocking the cognate Hsp receptor CD91 [161] on DC. Blocking of CD91 prior to co-incubation with α -TOS-derived tumor supernatant resulted in partial inhibition of co-stimulatory molecule expression [138, 147] and down-regulation of the antigen presenting machinery components (data not shown). HMGB-1 is a chromatin binding protein that is released by cells experiencing autophagy [162] or undergoing necrotic or apoptotic forms of cell death [163, 164] and which contributes to DC activation by binding to, and triggering the toll-like receptor-4 (TLR-4) signaling pathway [159]. We have recently demonstrated the presence of HMGB-1 in enriched autophagosome fractions and in the culture supernatant of tumor cells treated with α -TEA (data not shown). Whether or not DC activation and enhancement of cross-presentation are mediated through HMGB-1-TLR4 interactions remains to be verified in this system.

Concluding Remarks/Therapeutic Implications For Cancer

The relative absence of *in vivo* host toxicity of VEAs coupled with their immune-enhancing properties make this class of mitocans attractive targets for cancer treatment compared to current chemotherapeutic agents. Their selective toxicity towards tumor cells without causing measurable damage to the immune system is another desirable attribute of this class of mitocans. Cumulatively, the work from our laboratory has shown that α -TOS and α -TEA can: 1) suppress growth of established tumors and dramatically reduce the incidence of spontaneous metastases when combined with DC vaccination and 2) cause immunogenic tumor cell death which results in the release of “danger signals” that contribute to enhanced antigen cross-presentation and T cell effector function. The next important step in exploiting the utility of this novel class of mitocans as anti-cancer agents is to translate these laboratory findings to the clinic. Efforts are underway by our group to conduct animal toxicity studies on both α -TOS and α -TEA prior to initiating Phase I trials in humans. The knowledge that these mitocans can stimulate the immune system while directly killing tumor cells, suggests that they can be combined with additional immune modulators such as anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4 (ipilumimab), anti-programmed death ligand-1 (PDL-1), anti-programmed death-1 (PD-1) or small molecule inhibitors of TGF- β signaling (e.g. SM16) to overcome tolerance and tumor-mediated immune suppression respectively and further improve anti-tumor responses.

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Reference List

1. Srikrishna G, Freeze HH. Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. *Neoplasia*. 2009; 11(7):615–628. [PubMed: 19568407]
2. Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. Major cell death pathways at a glance. *Microbes Infect*. 2009; 11(13):1050–1062. [PubMed: 19733681]
3. Fink SL, Cookson BT. Apoptosis pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun*. 2005; 73(4):1907–1916. [PubMed: 15784530]
4. Kepp O, Tesniere A, Zitvogel L, Kroemer G. The immunogenicity of tumor cell death. *Curr Opin Oncol*. 2009; 21(1):71–76. [PubMed: 19125021]
5. Kepp O, Tesniere A, Schlemmer F, Michaud M, Senovilla L, Zitvogel L, Kroemer G. Immunogenic cell death modalities and their impact on cancer treatment. *Apoptosis*. 2009; 14(4):364–375. [PubMed: 19145485]
6. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. *Int J Cell Biol*. 2010; 2010:214074. [PubMed: 20182529]
7. Walker NI, Harmon BV, Gobe GC, Kerr JF. Patterns of cell death. *Methods Achiev Exp Pathol*. 1988; 13:18–54. [PubMed: 3045494]
8. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol*. 2009; 7(2):99–9109. [PubMed: 19148178]
9. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, Tasdemir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin NM, Haynes NM, Smyth MJ, Kroemer G, Zitvogel L. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1 β -dependent adaptive immunity against tumors. *Nat Med*. 2009; 15(10):1170–1178. [PubMed: 19767732]
10. Schroder K, Tschopp J. The inflammasomes. *Cell*. 2010; 140(6):821–832. [PubMed: 20303873]
11. Latz E. The inflammasomes: mechanisms of activation and function. *Curr Opin Immunol*. 2010; 22(1):28–33. [PubMed: 20060699]
12. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol*. 2009; 27:229–265. [PubMed: 19302040]
13. Bao Q, Shi Y. Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ*. 2007; 14(1):56–65. [PubMed: 16977332]
14. Teng X, Hardwick JM. The apoptosome at high resolution. *Cell*. 2010; 141(3):402–404. [PubMed: 20434981]
15. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*. 2004; 430(6996):213–218. [PubMed: 15190255]
16. Shaw PJ, Lamkanfi M, Kanneganti TD. NOD-like receptor (NLR) signaling beyond the inflammasome. *Eur J Immunol*. 2010; 40(3):624–627. [PubMed: 20201016]
17. Lamkanfi M, Dixit VM. Inflammasomes: guardians of cytosolic sanctity. *Immunol Rev*. 2009; 227(1):95–9105. [PubMed: 19120479]
18. Pedra JHF, Cassel SL, Sutterwala FS. Sensing pathogens and danger signals by the inflammasome. *Curr Opin Immunol*. 2009; 21(1):10–16. [PubMed: 19223160]
19. Martins I, Tesniere A, Kepp O, Michaud M, Schlemmer F, Senovilla L, Seror C, Metivier D, Perfettini JL, Zitvogel L, Kroemer G. Chemotherapy induces ATP release from tumor cells. *Cell Cycle*. 2009; 8(22):3723–3728. [PubMed: 19855167]
20. Kabakov AE, Gabai VL. Heat shock-induced accumulation of 70-kDa stress protein (HSP70) can protect ATP-depleted tumor cells from necrosis. *Exp Cell Res*. 1995; 217(1):15–21. [PubMed: 7867713]
21. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*. 1997; 185(8):1481–1486. [PubMed: 9126928]

22. Leist M, Single B, Naumann H, Fava E, Simon B, Kuhnle S, Nicotera P. Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. *Exp Cell Res.* 1999; 249(2):396–403. [PubMed: 10366439]
23. Tsujimoto Y, Shimizu S, Eguchi Y, Kamiike W, Matsuda H. Bcl-2 and Bcl-xL block apoptosis as well as necrosis: possible involvement of common mediators in apoptotic and necrotic signal transduction pathways. *Leukemia.* 1997; 11(3):380–382. [PubMed: 9209397]
24. Aymeric L, Apetoh L, Ghiringhelli F, Tesniere A, Martins I, Kroemer G, Smyth MJ, Zitvogel L. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. *Cancer Res.* 2010; 70(3):855–858. [PubMed: 20086177]
25. Kaufmann A, Musset B, Limberg SH, Renigunta V, Sus R, Dalpke AH, Heeg KM, Robaye B, Hanley PJ. “Host tissue damage” signal ATP promotes non-directional migration and negatively regulates toll-like receptor signaling in human monocytes. *J Biol Chem.* 2005; 280(37):32459–32467. [PubMed: 16030017]
26. Hanley PJ, Musset B, Renigunta V, Limberg SH, Dalpke AH, Sus R, Heeg KM, Preisig-Muller R, Daut J. Extracellular ATP induces oscillations of intracellular Ca²⁺ and membrane potential and promotes transcription of IL-6 in macrophages. *Proc Natl Acad Sci U S A.* 2004; 101(25):9479–9484. [PubMed: 15194822]
27. Elliott MR, Cheken FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, Lysiak JJ, Harden TK, Leitinger N, Ravichandran KS. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature.* 2009; 461(7261):282–286. [PubMed: 19741708]
28. Hornung V, Latz E. Intracellular DNA recognition. *Nat Rev Immunol.* 2010; 10(2):123–130. [PubMed: 20098460]
29. Lippmann J, Rothenburg S, Deigendesch N, Eitel J, Meixenberger K, van Laak V, Slevogt H, N'guessan PD, Hippenstiel S, Chakraborty T, Flieger A, Suttorp N, Opitz B. IFN β responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI). *Cell Microbiol.* 2008; 10(12):2579–2588. [PubMed: 18771559]
30. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, Ohba Y, Taniguchi T. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature.* 2007; 448(7152):501–505. [PubMed: 17618271]
31. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature.* 2009; 458(7237):509–513. [PubMed: 19158676]
32. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature.* 2009; 458(7237):514–518. [PubMed: 19158675]
33. Schroder K, Muruve DA, Tschopp J. Innate immunity: cytoplasmic DNA sensing by the AIM2 inflammasome. *Curr Biol.* 2009; 19(6):262–265.
34. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, Uematsu S, Takeuchi O, Takeshita F, Coban C, Akira S. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature.* 2008; 451(7179):725–729. [PubMed: 18256672]
35. Kaiser WJ, Upton JW, Mocarski ES. Receptor-interacting protein homotypic interaction motif-dependent control of NF- κ B activation via the DNA-dependent activator of IFN regulatory factors. *J Immunol.* 2008; 181(9):6427–6434. [PubMed: 18941233]
36. Rebsamen M, Heinz LX, Meylan E, Michallet MC, Schroder K, Hofmann K, Vazquez J, Benedict CA, Tschopp J. DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF- κ B. *EMBO Rep.* 2009; 10(8):916–922. [PubMed: 19590578]
37. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol.* 2009; 10(10):1065–1072. [PubMed: 19609254]
38. Chiu YH, Macmillan JB, Chen ZJ. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell.* 2009; 138(3):576–591. [PubMed: 19631370]
39. Choi MK, Wang Z, Ban T, Yanai H, Lu Y, Koshiba R, Nakaima Y, Hangai S, Savitsky D, Nakasato M, Negishi H, Takeuchi O, Honda K, Akira S, Tamura T, Taniguchi T. A selective

- contribution of the RIG-I-like receptor pathway to type I interferon responses activated by cytosolic DNA. *Proc Natl Acad Sci U S A*. 2009; 106(42):17870–17875. [PubMed: 19805092]
40. Yoneyama M, Fujita T. RNA recognition and signal transduction by RIG-I-like receptors. *Immunol Rev*. 2009; 227(1):54–65. [PubMed: 19120475]
 41. Bricio T, Molina A, Martin A, Mampaso F. In vitro modulation of interleukin-1 beta secretion by cultured rat doxorubicin-stimulated whole glomeruli and dissociated mesangial glomerular cells. *Immunology*. 1994; 81(1):53–57. [PubMed: 8132220]
 42. Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. *Cancer Res*. 2002; 62(16):4592–4598. [PubMed: 12183413]
 43. Thalappilly S, Sadasivam S, Radha V, Swarup G. Involvement of caspase 1 and its activator Ipaf upstream of mitochondrial events in apoptosis. *FEBS J*. 2006; 273(12):2766–2778. [PubMed: 16817903]
 44. Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, Jahn H, Planyavsky M, Bilban M, Colinge J, Bennett KL, Superti-Furga G. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol*. 2009; 10(3):266–272. [PubMed: 19158679]
 45. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, Hume DA, Stacey KJ. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science*. 2009; 323(5917):1057–1060. [PubMed: 19131592]
 46. Rathinam VAK, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol*. 2010; 11(5):395–402. [PubMed: 20351692]
 47. Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, Tschopp J. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature*. 2008; 452(7183):103–107. [PubMed: 18288107]
 48. Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, Nakasato M, Lu Y, Hangai S, Koshiba R, Savitsky D, Ronfani L, Akira S, Bianchi ME, Honda K, Tamura T, Kodama T, Taniguchi T. HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature*. 2009; 462(7269):99–103. [PubMed: 19890330]
 49. Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol*. 2010; 28:367–388. [PubMed: 20192808]
 50. Martinon F. Signaling by ROS drives inflammasome activation. *Eur J Immunol*. 2010; 40(3):616–619. [PubMed: 20201014]
 51. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol*. 2010; 10(3):210–215. [PubMed: 20168318]
 52. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science*. 2008; 320(5876):674–677. [PubMed: 18403674]
 53. van de Veerdonk FL, Smeekens SP, Joosten LAB, Kullberg BJ, Dinarello CA, van der Meer JWM, Netea MG. Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease. *Proc Natl Acad Sci U S A*. 2010; 107(7):3030–3033. [PubMed: 20133696]
 54. van BR, Koker MY, Jansen M, van HM, Roos D, Kuijpers TW, van den Berg TK. Human NLRP3 inflammasome activation is Nox1–4 independent. *Blood*. 2010; 115(26):5398–5400. [PubMed: 20407038]
 55. Emmendorffer A, Roesler J, Elsner J, Raeder E, Lohmann-Matthes ML, Meier B. Production of oxygen radicals by fibroblasts and neutrophils from a patient with x-linked chronic granulomatous disease. *Eur J Haematol*. 1993; 51(4):223–227. [PubMed: 8243611]
 56. Hu S, Zhao H, Yin XJ, Ma JKH. Role of mitochondria in silica-induced apoptosis of alveolar macrophages: inhibition of apoptosis by rhodamine 6G and N-acetyl-L-cysteine. *J Toxicol Environ Health A*. 2007; 70(17):1403–1415. [PubMed: 17687726]

57. Cruz CM, Rinna A, Forman HJ, Ventura ALM, Persechini PM, Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem*. 2007; 282(5):2871–2879. [PubMed: 17132626]
58. Franchi L, Eigenbrod T, Nunez G. Cutting edge: TNF- α mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol*. 2009; 183(2):792–796. [PubMed: 19542372]
59. Bowie A, O'Neill LA. Oxidative stress and nuclear factor- κ B activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol*. 2000; 59(1):13–23. [PubMed: 10605930]
60. Haddad JJ. Redox regulation of pro-inflammatory cytokines and IkappaB- α /NF- κ B nuclear translocation and activation. *Biochem Biophys Res Commun*. 2002; 296(4):847–856. [PubMed: 12200125]
61. Hwang YS, Jeong M, Park JS, Kim MH, Lee DB, Shin BA, Mukaida N, Ellis LM, Kim HR, Ahn BW, Jung YD. Interleukin-1 β stimulates IL-8 expression through MAP kinase and ROS signaling in human gastric carcinoma cells. *Oncogene*. 2004; 23(39):6603–6611. [PubMed: 15208668]
62. Woo CH, Eom YW, Yoo MH, You HJ, Han HJ, Song WK, Yoo YJ, Chun JS, Kim JH. Tumor necrosis factor- α generates reactive oxygen species via a cytosolic phospholipase A2-linked cascade. *J Biol Chem*. 2000; 275(41):32357–32362. [PubMed: 10934206]
63. Pfeiffer ZA, Guerra AN, Hill LM, Gavala ML, Prabhu U, Aga M, Hall DJ, Bertics PJ. Nucleotide receptor signaling in murine macrophages is linked to reactive oxygen species generation. *Free Radic Biol Med*. 2007; 42(10):1506–1516. [PubMed: 17448897]
64. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F. The P2 $\times$ 7 receptor: a key player in IL-1 processing and release. *J Immunol*. 2006; 176(7):3877–3883. [PubMed: 16547218]
65. Hancock JT. The role of redox mechanisms in cell signalling. *Mol Biotechnol*. 2009; 43(2):162–166. [PubMed: 19507067]
66. Rhee SG. Cell signaling. H₂O₂, a necessary evil for cell signaling. *Science*. 2006; 312(5782):1882–1883. [PubMed: 16809515]
67. Janssen-Heininger YMW, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, Finkel T, Stamler JS, Rhee SG, van der Vliet A. Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radic Biol Med*. 2008; 45(1):1–17. [PubMed: 18423411]
68. Murphy MP. Mitochondria--a neglected drug target. *Curr Opin Investig Drugs*. 2009; 10(10):1022–1024.
69. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol*. 2010; 11(2):136–140. [PubMed: 20023662]
70. Tattoli I, Carneiro LA, Jehanno M, Magalhaes JG, Shu Y, Philpott DJ, Arnoult D, Girardin SE. NLRX1 is a mitochondrial NOD-like receptor that amplifies NF- κ B and JNK pathways by inducing reactive oxygen species production. *EMBO Rep*. 2008; 9(3):293–300. [PubMed: 18219313]
71. Arnoult D, Soares F, Tattoli I, Castanier C, Philpott DJ, Girardin SE. An N-terminal addressing sequence targets NLRX1 to the mitochondrial matrix. *J Cell Sci*. 2009; 122(Pt 17):3161–3168. [PubMed: 19692591]
72. Moore CB, Bergstralh DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, Accavitti-Loper MA, Madden VJ, Sun L, Ye Z, Lich JD, Heise MT, Chen Z, Ting JPY. NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature*. 2008; 451(7178):573–577. [PubMed: 18200010]
73. Gertz M, Steegborn C. The Lifespan-Regulator p66Shc in Mitochondria: Redox Enzyme or Redox Sensor? *Antioxid Redox Signal*. 2010
74. Tal MC, Sasai M, Lee HK, Yordy B, Shadel GS, Iwasaki A. Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. *Proc Natl Acad Sci U S A*. 2009; 106(8):2770–2775. [PubMed: 19196953]
75. Scott I, Norris KL. The mitochondrial antiviral signaling protein, MAVS, is cleaved during apoptosis. *Biochem Biophys Res Commun*. 2008; 375(1):101–106. [PubMed: 18692023]

76. Lei Y, Moore CB, Liesman RM, O'Connor BP, Bergstralh DT, Chen ZJ, Pickles RJ, Ting JPY. MAVS-mediated apoptosis and its inhibition by viral proteins. *PLoS One*. 2009; 4(5):e5466. [PubMed: 19404494]
77. Yu CY, Chiang RL, Chang TH, Liao CL, Lin YL. The interferon stimulator mitochondrial antiviral signaling protein facilitates cell death by disrupting the mitochondrial membrane potential and by activating caspases. *J Virol*. 2010; 84(5):2421–2431. [PubMed: 20032188]
78. Neuzil J, Tomasetti M, Zhao Y, Dong LF, Birringer M, Wang XF, Low P, Wu K, Salvatore BA, Ralph SJ. Vitamin E analogs, a novel group of “mitocans,” as anticancer agents: the importance of being redox-silent. *Mol Pharmacol*. 2007; 71(5):1185–1199. [PubMed: 17220355]
79. Neuzil J, Dong LF, Ramanathapuram L, Hahn T, Chladova M, Wang XF, Zobalova R, Prochazka L, Gold M, Freeman R, Turanek J, Akporiaye ET, Dyason JC, Ralph SJ. Vitamin E analogues as a novel group of mitocans: anti-cancer agents that act by targeting mitochondria. *Mol Aspects Med*. 2007; 28(5-6):607–645. [PubMed: 17499351]
80. Weber T, Dalen H, Andera L, Negre-Salvayre A, Auge N, Sticha M, Lloret A, Terman A, Witting PK, Higuchi M, Plasilova M, Zivny J, Gellert N, Weber C, Neuzil J. Mitochondria play a central role in apoptosis induced by alpha-tocopheryl succinate, an agent with antineoplastic activity: comparison with receptor-mediated pro-apoptotic signaling. *Biochemistry*. 2003; 42(14):4277–4291. [PubMed: 12680782]
81. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother Pharmacol*. 2004; 53(3):209–219. [PubMed: 14610616]
82. Wang XF, Dong L, Zhao Y, Tomasetti M, Wu K, Neuzil J. Vitamin E analogues as anticancer agents: lessons from studies with alpha-tocopheryl succinate. *Mol Nutr Food Res*. 2006; 50(8):675–685. [PubMed: 16835868]
83. Kogure K, Hama S, Manabe S, Tokumura A, Fukuzawa K. High cytotoxicity of alpha-tocopheryl hemisuccinate to cancer cells is due to failure of their antioxidative defense systems. *Cancer Lett*. 2002; 186(2):151–156. [PubMed: 12213284]
84. Wang XF, Witting PK, Salvatore BA, Neuzil J. Vitamin E analogs trigger apoptosis in HER2/erbB2-overexpressing breast cancer cells by signaling via the mitochondrial pathway. *Biochem Biophys Res Commun*. 2005; 326(2):282–289. [PubMed: 15582575]
85. Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem*. 2001; 276(7):4588–4596. [PubMed: 11092892]
86. Alleva R, Tomasetti M, Andera L, Gellert N, Borghi B, Weber C, Murphy MP, Neuzil J. Coenzyme Q blocks biochemical but not receptor-mediated apoptosis by increasing mitochondrial antioxidant protection. *FEBS Lett*. 2001; 503(1):46–50. [PubMed: 11513852]
87. Kang YH, Lee E, Choi MK, Ku JL, Kim SH, Park YG, Lim SJ. Role of reactive oxygen species in the induction of apoptosis by alpha-tocopheryl succinate. *Int J Cancer*. 2004; 112(3):385–392. [PubMed: 15382062]
88. Dong LF, Swettenham E, Eliasson J, Wang XF, Gold M, Medunic Y, Stantic M, Low P, Prochazka L, Witting PK, Turanek J, Akporiaye ET, Ralph SJ, Neuzil J. Vitamin E analogues inhibit angiogenesis by selective induction of apoptosis in proliferating endothelial cells: the role of oxidative stress. *Cancer Res*. 2007; 67(24):11906–11913. [PubMed: 18089821]
89. Dong LF, Freeman R, Liu J, Zobalova R, Marin-Hernandez A, Stantic M, Rohlena J, Valis K, Rodriguez-Enriquez S, Butcher B, Goodwin J, Brunk UT, Witting PK, Moreno-Sanchez R, Scheffler IE, Ralph SJ, Neuzil J. Suppression of tumor growth in vivo by the mitocan alpha-tocopheryl succinate requires respiratory complex II. *Clin Cancer Res*. 2009; 15(5):1593–1600. [PubMed: 19223492]
90. D'Alessio M, De NM, Coppola S, Gualandi G, Pugliese L, Cerella C, Cristofanon S, Civitareale P, Ciriolo MR, Bergamaschi A, Magrini A, Ghibelli L. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. *FASEB J*. 2005; 19(11):1504–1506. [PubMed: 15972297]

91. Dewson G, Kratina T, Czabotar P, Day CL, Adams JM, Kluck RM. Bak activation for apoptosis involves oligomerization of dimers via their alpha6 helices. *Mol Cell*. 2009; 36(4):696–703. [PubMed: 19941828]
92. Nie C, Tian C, Zhao L, Petit PX, Mehrpour M, Chen Q. Cysteine 62 of Bax is critical for its conformational activation and its proapoptotic activity in response to H₂O₂-induced apoptosis. *J Biol Chem*. 2008; 283(22):15359–15369. [PubMed: 18344566]
93. Prochazka L, Dong LF, Valis K, Freeman R, Ralph SJ, Turanek J, Neuzil J. alpha-Tocopheryl succinate causes mitochondrial permeabilization by preferential formation of Bak channels. *Apoptosis*. 2010; 15(7):782–794. [PubMed: 20217235]
94. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001; 410(6832):1107–1111. [PubMed: 11323675]
95. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol*. 2006; 90:1–50. [PubMed: 16730260]
96. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*. 2004; 21(2):137–148. [PubMed: 15308095]
97. Swann JB, Smyth MJ. Immune surveillance of tumors. *J Clin Invest*. 2007; 117(5):1137–1146. [PubMed: 17476343]
98. Gattinoni L, Powell DJ, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol*. 2006; 6(5):383–393. [PubMed: 16622476]
99. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998; 392(6673):245–252. [PubMed: 9521319]
100. Boczkowski D, Nair SK, Nam JH, Lysterly HK, Gilboa E. Induction of tumor immunity and cytotoxic T lymphocyte responses using dendritic cells transfected with messenger RNA amplified from tumor cells. *Cancer Res*. 2000; 60(4):1028–1034. [PubMed: 10706120]
101. Smits ELJM, Ponsaerts P, Berneman ZN, Van Tendeloo VFI. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. *Oncologist*. 2008; 13(8):859–875. [PubMed: 18701762]
102. Smyth MJ, Cretney E, Kershaw MH, Hayakawa Y. Cytokines in cancer immunity and immunotherapy. *Immunol Rev*. 2004; 202:275–293. [PubMed: 15546400]
103. Cheuk ATC, Mufti GJ, Guinn Ba. Role of 4-1BB:4-1BB ligand in cancer immunotherapy. *Cancer Gene Ther*. 2004; 11(3):215–226. [PubMed: 14671675]
104. Sugamura K, Ishii N, Weinberg AD. Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40. *Nat Rev Immunol*. 2004; 4(6):420–431. [PubMed: 15173831]
105. Schabowsky RH, Madireddi S, Sharma R, Yolcu ES, Shirwan H. Targeting CD4+CD25+FoxP3+ regulatory T-cells for the augmentation of cancer immunotherapy. *Curr Opin Investig Drugs*. 2007; 8(12):1002–1008.
106. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, Rietz C, Flies DB, Lau JS, Zhu G, Tamada K, Chen L. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res*. 2005; 65(3):1089–1096. [PubMed: 15705911]
107. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 1996; 271(5256):1734–1736. [PubMed: 8596936]
108. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004; 10(9):909–915. [PubMed: 15340416]
109. Bodey B, Bodey B, Siegel SE, Kaiser HE. Immunocytochemical detection of MMP-3 and -10 expression in hepatocellular carcinomas. *Anticancer Res*. 2000; 20(6B):4585–4590. [PubMed: 11210857]
110. Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, Duray PH, Steinberg SM, Allison JP, Davis TA, Rosenberg SA. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*. 2003; 100(14):8372–8377. [PubMed: 12826605]

111. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccocanti F, Piacentini M, Zitvogel L, Kroemer G. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med.* 2007; 13(1):54–61. [PubMed: 17187072]
112. Lake RA, Robinson BWS. Immunotherapy and chemotherapy--a practical partnership. *Nat Rev Cancer.* 2005; 5(5):397–405. [PubMed: 15864281]
113. Liu JY, Wu Y, Zhang XS, Yang JL, Li HL, Mao YQ, Wang Y, Cheng X, Li YQ, Xia JC, Masucci M, Zeng YX. Single administration of low dose cyclophosphamide augments the antitumor effect of dendritic cell vaccine. *Cancer Immunol Immunother.* 2007; 56(10):1597–1604. [PubMed: 17440723]
114. Apetoh L, Mignot G, Panaretakis T, Kroemer G, Zitvogel L. Immunogenicity of anthracyclines: moving towards more personalized medicine. *Trends Mol Med.* 2008; 14(4):141–151. [PubMed: 18353726]
115. Zitvogel L, Kepp O, Senovilla L, Menger L, Chaput N, Kroemer G. Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. 2010; 2010/04/28:3100–3104.
116. Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. 2007; 2007/02/15:4839–4845.
117. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. 1982; 1982/04/01:1063–1074.
118. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. 2005; 2004/12/14:2862–2868.
119. Fulda S, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. 2010; 2010/05/15:447–464.
120. Deaglio S, Canella D, Baj G, Arnulfo A, Waxman S, Malavasi F. Evidence of an immunologic mechanism behind the therapeutical effects of arsenic trioxide (As(2)O(3)) on myeloma cells. 2001; 2001/02/28:227–235.
121. Thejass P, Kuttan G. Augmentation of natural killer cell and antibody-dependent cellular cytotoxicity in BALB/c mice by sulforaphane, a naturally occurring isothiocyanate from broccoli through enhanced production of cytokines IL-2 and IFN-gamma. 2006; 2006/09/26:443–457.
122. Robinson E, Rubin D, Mekori T, Segal R, Pollack S. In vivo modulation of natural killer cell activity by tamoxifen in patients with bilateral primary breast cancer. 1993; 1993/08/01:209–212.
123. Rotstein S, Blomgren H, Petrini B, Wasserman J, von Stedingk LV. Influence of adjuvant tamoxifen on blood lymphocytes. 1988; 1988/09/01:75–79.
124. Yang Y, Paik JH, Cho D, Cho JA, Kim CW. Resveratrol induces the suppression of tumor-derived CD4+CD25+ regulatory T cells. 2008; 2008/03/11:542–547.
125. Behjati S, Frank MH. The effects of tamoxifen on immunity. 2009; 2009/08/20:3076–3080.
126. Nizar S, Copier J, Meyer B, Bodman-Smith M, Galustian C, Kumar D, Dalgleish A. T-regulatory cell modulation: the future of cancer immunotherapy? 2009; 2009/04/23:1697–1703.
127. Sharma S, Chopra K, Kulkarni SK, Agrewala JN. Resveratrol and curcumin suppress immune response through CD28/CTLA-4 and CD80 co-stimulatory pathway. 2007; 2006/12/21:155–163.
128. Svajger U, Obermajer N, Jeras M. Dendritic cells treated with resveratrol during differentiation from monocytes gain substantial tolerogenic properties upon activation. 2010; 2009/12/17:525–535.
129. Singh NP, Hegde VL, Hofseth LJ, Nagarkatti M, Nagarkatti P. Resveratrol (trans-3,5,4-trihydroxystilbene) ameliorates experimental allergic encephalomyelitis, primarily via induction of apoptosis in T cells involving activation of aryl hydrocarbon receptor and estrogen receptor. 2007; 2007/09/18:1508–1521.
130. Udenigwe CC, Ramprasath VR, Aluko RE, Jones PJ. Potential of resveratrol in anticancer and anti-inflammatory therapy. 2008; 2008/08/01:445–454.

131. Anderson K, Simmons-Menchaca M, Lawson KA, Atkinson J, Sanders BG, Kline K. Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E Succinate and vitamin E analogue, alpha-TEA. *Cancer Res.* 2004; 64(12):4263–4269. [PubMed: 15205340]
132. Hahn T, Szabo L, Gold M, Ramanathapuram L, Hurley LH, Akporiaye ET. Dietary administration of the proapoptotic vitamin E analogue alpha-tocopheryloxyacetic acid inhibits metastatic murine breast cancer. *Cancer Res.* 2006; 66(19):9374–9378. [PubMed: 17018590]
133. Hahn T, Fried K, Hurley LH, Akporiaye ET. Orally active alpha-tocopheryloxyacetic acid suppresses tumor growth and multiplicity of spontaneous murine breast cancer. *Mol Cancer Ther.* 2009; 8(6):1570–1578. [PubMed: 19509249]
134. Lawson KA, Anderson K, Simmons-Menchaca M, Atkinson J, Sun L, Sanders BG, Kline K. Comparison of vitamin E derivatives alpha-TEA and VES in reduction of mouse mammary tumor burden and metastasis. *Exp Biol Med (Maywood).* 2004; 229(9):954–963. [PubMed: 15388892]
135. Lawson KA, Anderson K, Snyder RM, Simmons-Menchaca M, Atkinson J, Sun LZ, Bandyopadhyay A, Knight V, Gilbert BE, Sanders BG, Kline K. Novel vitamin E analogue and 9-nitro-camptothecin administered as liposome aerosols decrease syngeneic mouse mammary tumor burden and inhibit metastasis. *Cancer Chemother Pharmacol.* 2004; 54(5):421–431. [PubMed: 15197487]
136. Malafa MP, Fokum FD, Mowlavi A, Abusief M, King M. Vitamin E inhibits melanoma growth in mice. *Surgery.* 2002; 131(1):85–91. [PubMed: 11812968]
137. Neuzil J, Weber T, Gellert N, Weber C. Selective cancer cell killing by alpha-tocopheryl succinate. *Br J Cancer.* 2001; 84(1):87–89. [PubMed: 11139318]
138. Ramanathapuram LV, Hahn T, Graner MW, Katsanis E, Akporiaye ET. Vesiculated alpha-tocopheryl succinate enhances the anti-tumor effect of dendritic cell vaccines. *Cancer Immunol Immunother.* 2006; 55(2):166–177. [PubMed: 16041582]
139. Kogure K, Manabe S, Hama S, Tokumura A, Fukuzawa K. Potentiation of anti-cancer effect by intravenous administration of vesiculated alpha-tocopheryl hemisuccinate on mouse melanoma in vivo. *Cancer Lett.* 2003; 192(1):19–24. [PubMed: 12637149]
140. Ramanathapuram LV, Kobie JJ, Bearss D, Payne CM, Trevor KT, Akporiaye ET. alpha-Tocopheryl succinate sensitizes established tumors to vaccination with nonmatured dendritic cells. *Cancer Immunol Immunother.* 2004; 53(7):580–588. [PubMed: 14991239]
141. Neuzil J. Vitamin E succinate and cancer treatment: a vitamin E prototype for selective antitumour activity. *Br J Cancer.* 2003; 89(10):1822–1826. [PubMed: 14612885]
142. Neuzil J, Tomasetti M, Mellick AS, Alleva R, Salvatore BA, Birringer M, Fariss MW. Vitamin E analogues: a new class of inducers of apoptosis with selective anti-cancer effects. *Curr Cancer Drug Targets.* 2004; 4(4):355–372. [PubMed: 15180501]
143. Tomasetti M, Strafella E, Staffolani S, Santarelli L, Neuzil J, Guerrieri R. alpha-Tocopheryl succinate promotes selective cell death induced by vitamin K3 in combination with ascorbate. *Br J Cancer.* 2010; 102(8):1224–1234. [PubMed: 20332775]
144. Kline K, Yu W, Sanders BG. Vitamin E: mechanisms of action as tumor cell growth inhibitors. *J Nutr.* 2001; 131(1):163.
145. Neuzil J. Alpha-tocopheryl succinate epitomizes a compound with a shift in biological activity due to pro-vitamin-to-vitamin conversion. *Biochem Biophys Res Commun.* 2002; 293(5):1309–1313. [PubMed: 12054655]
146. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. *Nat Rev Immunol.* 2008; 8(1):59–73. [PubMed: 18097448]
147. Ramanathapuram LV, Hahn T, Dial SM, Akporiaye ET. Chemo-immunotherapy of breast cancer using vesiculated alpha-tocopheryl succinate in combination with dendritic cell vaccination. *Nutr Cancer.* 2005; 53(2):177–193. [PubMed: 16573379]
148. Heufler C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, Enk A, Steinman RM, Romani N, Schuler G. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol.* 1996; 26(3):659–668. [PubMed: 8605935]
149. Kline K, Yu W, Sanders BG. Vitamin E and breast cancer. *J Nutr.* 2004; 134(12 Suppl):3462.

150. Kline K, Lawson KA, Yu W, Sanders BG. Vitamin E and cancer. *Vitam Horm.* 2007; 76:435–461. [PubMed: 17628185]
151. Neuzil J, Dyason JC, Freeman R, Dong LF, Prochazka L, Wang XF, Scheffler I, Ralph SJ. Mitocans as anti-cancer agents targeting mitochondria: lessons from studies with vitamin E analogues, inhibitors of complex II. *J Bioenerg Biomembr.* 2007; 39(1):65–72. [PubMed: 17294131]
152. Feng H, Zeng Y, Graner MW, Katsanis E. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood.* 2002; 100(12):4108–4115. [PubMed: 12393401]
153. Srivastava P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu Rev Immunol.* 2002; 20:395–425. [PubMed: 11861608]
154. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol.* 2000; 12(11):1539–1546. [PubMed: 11058573]
155. Bethke K, Staib F, Distler M, Schmitt U, Jonuleit H, Enk AH, Galle PR, Heike M. Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. *J Immunol.* 2002; 169(11):6141–6148. [PubMed: 12444117]
156. Flohe SB, Bruggemann J, Lendemans S, Nikulina M, Meierhoff G, Flohe S, Kolb H. Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. *J Immunol.* 2003; 170(5):2340–2348. [PubMed: 12594256]
157. Basu S, Srivastava PK. Fever-like temperature induces maturation of dendritic cells through induction of hsp90. *Int Immunol.* 2003; 15(9):1053–1061. [PubMed: 12917257]
158. Mosser DD, Morimoto RI. Molecular chaperones and the stress of oncogenesis. *Oncogene.* 2004; 23(16):2907–2918. [PubMed: 15077153]
159. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med.* 2007; 13(9):1050–1059. [PubMed: 17704786]
160. Chu Y, Wang LX, Yang G, Ross HJ, Urba WJ, Prell R, Jooss K, Xiong S, Hu HM. Efficacy of GM-CSF-producing tumor vaccine after docetaxel chemotherapy in mice bearing established Lewis lung carcinoma. *J Immunother.* 2006; 29(4):367–380. [PubMed: 16799332]
161. Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity.* 2001; 14(3):303–313. [PubMed: 11290339]
162. Livesey KM, Tang D, Zeh HJ, Lotze MT. Autophagy inhibition in combination cancer treatment. *Curr Opin Investig Drugs.* 2009; 10(12):1269–1279.
163. Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity.* 2008; 29(1):21–32. [PubMed: 18631454]
164. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature.* 2002; 418(6894):191–195. [PubMed: 12110890]

List of Abbreviations

-TEA	-tocopheryloxyacetic acid
-TOH	-tocopherol
-TOS	alpha-tocopheryl succinate
AIM2	absent in melanoma 2
Apaf-1	apoptotic protease activating factor 1

ASC	apoptosis-associated speck-like protein
BAX	BCL-2 associated $\times$ protein
BCL-2	B cell lymphoma/leukemia 2
BCL-xL	BCL-2-like large protein
BH3	BCL-2 homology domain 3
CAD	cytoplasmic Dnase
CARD	caspase recruitment domain
CGD	chronic granulomatous disease
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte associated protein 4
DAI	DNA-dependent activator of IRF
DAMP	damage-associated molecular patterns
DMSO	dimethylsulfoxide
DC	dendritic cell
DISC	death-inducing signaling complex
DPI	diphenylene iodonium
ERK1/2	extracellular signal-regulated kinase 1/2
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIN-200	hemopoietic IFN-inducible nuclear protein 200
HMGB1	high mobility group protein 1
Hsp	heat shock protein
ICE	interleukin converting enzyme
IFN	type-I interferon
IFN-	interferon beta
IFN-	interferon gamma
IL	interleukin
IPAF	ICE protease-activating factor
JNK	c-Jun N-terminal kinase
FMF	familial mediterranean fever
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAVS	mitochondrial antiviral signalling protein
MDA-5	melanoma differentiation-associated gene-5
MHC	Class I major histocompatibility complex I
Mito Q	mitoquinone
MOMP	mitochondrial outer membrane permeability

NACHT	neuronal apoptosis inhibitor protein, C2TA, HET-E and TP1
NF- B	nuclear factor kappa B
NLR	NACHT or NOD-like receptor
NLRP3	NACHT, LRR and PYD domain-containing protein 3 (also known as NALP3)
NLRX1	Nod-like receptor family
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular patterns
PI3K	phosphoinositide 3 kinase
PD-1	programmed death-1 protein
PDL-1	programmed death protein ligand-1
PRR	pattern recognition receptors
PYD	PYRIN domain
RAGE	receptor for advanced glycation end products
RHIM	RIP homotypic interaction motif
RIG-1	retinoid-inducible gene 1
RIP	receptor-interacting protein
RLR	RIG-I-like receptors
ROG-I	retinoic acid-inducible gene-I
ROS	reactive oxygen species
SDH	succinate dehydrogenase
siRNA	small interfering RNA
SOD	superoxide dismutase
TANK	TRAF family member-associated NF- B activator
TBK-1	TANK binding kinase 1
TGF-	transforming growth factor beta
TH₁	T helper 1
TLR	toll like receptor
TNF-	tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRX	thioredoxin
TTFA	thenoyltrifluoroacetone
TXNIP	thioredoxin-interacting protein
UbQ	ubiquinone
UQCRC2	ubiquinol cytochrome reductase
VEA	vitamin E analog

VES vitamin E succinate

Fig. (1). A. Structural domains in representative examples of the inflammasomes: AIM; NLRP3 (NALP3) and IPAF

The inflammasomes include a superfamily of multi-domain proteins that on ligand binding provide scaffolds for the assembly of large oligomeric complexes resulting in the activation of caspases that then cleave pro-inflammatory cytokines pro-IL-1 β and -IL-18 into the mature form for secretion to promote the immune response. The multiple domains in their structure can include: leucine-rich-repeats (LRR); HIN-200 like DNA binding domains (HIN); Nucleotide binding and oligomerization domain (NOD); PYRIN like domains (PYD); and Caspase recruitment domain (CARD). **B. Insert:** Structural domains of the DNA-dependent activator of IRFs (DAI) [26, 27].

Fig. (2).
Diagram indicating the cytosolic DNA sensors, AIM2 and DAI and their function in cells as pro-inflammatory signalling agents.

Figure 3.

Structure of vitamin E (RRR- α -tocopherol, α -TOH, **A**) and the vitamin E derivatives RRR- α -tocopheryl succinate (α -TOS, **B**) and RRR- α -tocopheryloxy acetic acid (α -TEA, **C**). Alpha-TOS possesses an ester-linked succinate moiety attached to carbon number 6 of the chromanol head, making it susceptible to hydrolysis and cleavage by esterases. In contrast, α -TEA possesses an ether-linked acetic acid moiety attached to the carbon number 6 of the chromanol head rendering α -TEA resistant to cleavage by esterases.

Figure 4. Effect of α-TOS on the function of immune cells *in vivo*

Mice received 3 intraperitoneal injections of α-TOS (4 mg per injection) or vehicle (Ethanol) four days apart. Forty-eight hours after the last injection, DC or CD4⁺ T cells were enriched from pooled splenocytes from 3 mice. **(A)** IL-12p70 secretion by DC was determined by ELISA after *in vitro* stimulation with 20ng/ml TNF-α for 24 hours. **(B)** CD4⁺ T cells were stimulated *in vitro* with the mitogen Concanavalin A (ConA) for 4 days and proliferation was determined by [³H]-thymidine incorporation. The values represent mean ± SD of triplicate samples.

Figure 5. Efficacy of the combination of α-TEA and DC against spontaneous murine breast cancer

(A) Transgenic MMTV-PyMT mice that spontaneously develop up to 10 breast carcinomas received α-TEA in the diet (~2mg/day) starting at 6 weeks of age (day 42) until 15 weeks of age (day 105). Three s.c. injections of 10^6 non-matured, bone marrow-derived DC were given at weeks 8, 9 and 10. The values represent the cumulative tumor areas per mouse on day 105. Boxed number: average cumulative tumor area ± SEM. (B) Average tumors per mouse (tumor multiplicity) calculated as: Tumor multiplicity = [# tumors per group]/[# of mice per group]. Multiplicity curves were determined by non-linear regression. Boxed numbers: tumor multiplicity ± SEM.

Figure 6. Effect of V α -TOS-derived tumor cell supernatant on endocytosis and phagocytosis by DC

Non-matured DC were incubated at 37°C with supernatant derived from tumor cells treated with V α -TOS (V α -TOS_s), PBS (PBS_s) or TNF- α or left untreated for 24 hours. The phagocytic and endocytic properties of the DC were evaluated by determining the ability to take up FITC-conjugated dextran beads (endocytosis) and FITC-conjugated *E.coli* particles (phagocytosis) at 4°C and at 37°C using flow cytometry. The change of mean fluorescence intensity (DMFI) was calculated as DMFI=[(MFI at 37°C)-(MFI at 4°C)]. Data represent mean DMFI $\pm$ SD of MFI values from 3 independent experiments. Cells were gated on light scatter and CD11c-expression.

Figure 7. V α -TOS-derived tumor cell supernatant increases expression of antigen processing machinery (APM) components by DC

DC were incubated for 24 hours with supernatant derived from V α -TOS (V α -TOS_S), PBS-treated (PBS_S) tumor cells or with TNF- α and then stained with delta-specific mAb (SY-5), ERp 57-specific mAb (T O-3) and tapasin-specific mAb (TO-2) by intracellular staining and analyzed by flow cytometry. Surface expression of MHC Class I antigens was determined by staining with a PE-conjugated H2D^d-specific antibody. Cells were gated on light scatter and CD11c-expression.

Figure 8. -TEA induces mitochondrial depolarization and production of reactive oxygen species
(A) 4T1 breast tumor cells were treated with the indicated concentrations of V -TEA for 6 hours and subsequently mitochondrial membrane potential was evaluated by staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and flow cytometry. Data represent fold change of percent monomeric JC-1-stained cells (normalized to untreated cells) $\pm$ SEM of three independent experiments. **(B)** 4T1 tumor cells were treated with 80 μ M V -TEA for the indicated time periods and the accumulation of reactive oxygen species (ROS) was determined by 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) stain and flow cytometry. Data represent fold change of mean fluorescence intensity of DCFDA stained cells (normalized to untreated cells) $\pm$ SEM of three independent experiments.

Figure 9. Schematic representation of the immune modulatory effects of VEAs

VEAs modulate several pathways associated with the generation of antigen-specific immune response but the initial events seem to be directly connected with the selective pro-apoptotic properties of VEAs towards tumor cells. In this scenario VEAs target mitochondria of tumor cells and induce the accumulation of reactive oxygen species (ROS). This mitochondrial damage may stimulate the caspase cascade and other pathways, ultimately leading to apoptotic tumor cell death promoting the release of putative tumor antigens. Autophagic tumor cell death and necrosis may also play a role. VEAs also act as adjuvants at all stages of antigen presentation starting from the mobilization of immature DC by up-regulating or inducing the release of heat shock proteins (Hsp) Hsp60, Hsp70 and Hsp90 and other factors such as HMGB-1 which help “sense” dying tumor cells. VEAs also increase antigen uptake and up-regulate proteins associated with the antigen-presenting machinery (APM) including proteins belonging to the multi-catalytic proteasome complex (delta) as well as the MHC class I loading complex (tapasin, ERp57). In addition, VEAs may enhance the expression of co-stimulatory molecules (CD40, CD80, CD86) on DC and the ability of DC to migrate toward chemokines attracting matured DC (mDC) to lymphatic organs where they encounter naïve T cells and antigen cross-presentation to CD8⁺ T cells occurs. The VEA-induced phenotypic changes in DC are associated with increased production of IL-12, which are essential for stimulation of naïve T cells and skewing the immune response toward T_H1. The activated tumor specific T cells can then attack tumor tissue and metastases.

Table I

Class	Name	Activity/Target	Modulation of anti-tumor immune response
Hexokinase inhibitors	3-bromopyruvate	Chen et al. Biochim Biophys Acta., 2009, 1787, 553-60; Xu et al., Cancer Res., 2005, 65(2),613-21	unknown
	2-deoxyglucose	Ko et al., Cancer Lett. 2001, 173(1),83-91; Simons et al Cancer Res., 2007, 67(7),3364-70	unknown
Bcl-2/Bcl-x _L mimetics	Gossypol	Kitada et al., J Med Chem., 2003, 46(20),4259-64	unknown
	Antimycin A	Tzung et al., Nat Cell Biol., 2001, (2),183-91	unknown
	Vitamin E analogs (- tocopheryl succinate)	Shiau et al., J Biol Chem., 2006, 281(17),11819-25	Ramanthapuram et al., Cancer Immunol Immunother., 2006, 55(2), 166-77; Ramanthapuram et al., Nutr Cancer., 2005, 53(2), 177-93
Thiol redox inhibitors	Isothiocyanates	Xu et al., Br J Cancer., 2001, 84(5),670-3	Thejass et al., Immunopharmacol Immunotoxicol., 2006, 28(3), 442-57
	Arsonic trioxide	Miller et al., Cancer Res., 2002, 62(14),3893-903	Deaglio et al., Leuk Res., 2001, 25(3), 227-35
Voltage dependent anion channel and/or adenine	Lonidamine	Oudard et al., J Neurooncol., 2003., 20(52),7579-87	unknown
	Arsenites	Belzaq et al., Oncogene., 2001, 20(52),7579-87	unknown
	Steroid analogues(CD437)	Marchetti et al., Cancer Res., 1999, 59(24),6257-66; Belzaq et al., Oncogene., 2001, 20(52),7579-87	unknown
Electrontransport chain targeting drugs	4-OH retinamide	Hail et al., J Biol Chem., 2001, 276(49),45614-21	unknown
	Tamoxifen	Moroira et al., J Biol Chem., 2006, 281(15),10143-52	Robinson et al. Cancer Immunol Immunother., 1993, 37(3), 209-12
	Antimycin A	Wolvetang et al., FEBS Lett., 1994, 339,40-4.	unknown
	Vitamin E analogs (- tocopheryl succinate)	Dong et al., Oncogene., 2008, 27(31), 4324-35	see above
Lipophilic targeting inner membrane	Rhodamine-123	Lampidis et al., Biochemistry., 2001, 40(18), 5542-7	unknown
	F16	Fantin et al. Cancer Cell., 2002, 2(1),29-42	unknown
	(KLAKKLAK) ₂ peptide	Elerby et al., Acta Biochim Pol., 2006:53(4),801-5	unknown
Drugs targeting other (unknown sites)	Resveratrol	Gledhill et al. PNAS., 2007, 104(34), 13632-7	Yang et al., Int Immunopharmacol., 2008, 8(4), 542-7

Class	Name	Activity/Target	Modulation of anti-tumor immune response
	Botulinic acid	Fulda et al., Cancer Res., 1997, 57(21):4956-64	unknown
	Parthenolide	Guzman et al., Blood., 2005, 1105(11):4163-9	unknown