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Molecular Pathways: Immunosuppressive Roles of IRE1α–XBP1 Signaling in Dendritic Cells of the Tumor Microenvironment

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Abstract

The endoplasmic reticulum (ER) is a massive cytoplasmic membrane network that functions primarily to ensure proper folding and post-translational modification of newly synthesized secretory and transmembrane proteins. Abnormal accumulation of unfolded proteins in this organelle causes a state of "ER stress", which is a hallmark feature of various diseases including cancer, neurodegeneration and metabolic dysfunction. Cancer cells exploit the IRE1α-XBP1 arm of the ER stress response to efficiently adjust their protein-folding capacity and ensure survival under hostile tumor microenvironmental conditions. However, we recently found that dendritic cells (DCs) residing in the ovarian cancer microenvironment also experience sustained ER stress and demonstrate persistent activation of the IRE1α-XBP1 pathway. This previously unrecognized process disrupts metabolic homeostasis and antigen-presenting capacity in DCs, thereby crippling their natural ability to support the protective function of infiltrating anti-tumor T cells. In this review, we briefly discuss some of the mechanisms that fuel ER stress in tumor-associated DCs, the biological processes altered by aberrant IRE1α-XBP1 signaling in these innate immune cells, and the unique immunotherapeutic potential of targeting this pathway in cancer hosts.

BACKGROUND

Triggering IRE1α-XBP1 activation through the ER stress response

The endoplasmic reticulum (ER) is the primary organelle responsible for regulating intracellular calcium, lipid biosynthesis, and the proper glycosylation and folding of nascent transmembrane and secreted proteins. Numerous physiological stimuli often found within tumor microenvironments such as nutrient deprivation, calcium store depletion, oxidative stress, hypoxia, and inflammation can disrupt the protein folding capacity of the ER. When this intrinsic protein folding capacity is overwhelmed, the cell is considered to be in a state

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of “ER stress” and will initiate an unfolded protein response (UPR) via the ER transmembrane proteins IRE1α (encoded by *Ern1*), PERK (encoded by *Eif2ak3*), and ATF6 (encoded by *Atf6*) in an attempt to restore homeostasis (1). If the combined action of these three proteins is insufficient to ameliorate ER toxicity, the affected cell will undergo apoptosis.

The serine/threonine-protein kinase/endoribonuclease IRE1α represents the most ancient branch of this signaling pathway, and is highly conserved from yeast to humans. At steady state, the chaperone protein BiP holds IRE1α in its monomeric form, thereby precluding activation. However, upon the induction of ER stress, the accumulating misfolded proteins titrate BiP away from IRE1α, triggering IRE1α dimerization, autophosphorylation, and a conformational shift that licenses its C-terminal endoribonuclease domain to cytoplasmically cleave 26 nucleotides from the *Xbp1* mRNA. This spliced transcript is subsequently re-ligated by the tRNA ligase RtcB (2), resulting in a critical reading frame shift that enables translation of the functionally active X-box binding protein 1 (XBP1). This multi-tasking transcription factor alleviates ER stress by upregulating a variety of chaperones, redox-dependent foldases, and glycosyltransferases. Beyond these canonical functions, several groups have demonstrated that XBP1 also modulates ER stress-independent, context-specific signaling events such as the hypoxia response (by dimerizing with HIF1α) (3), lipid metabolism (4), estrogen receptor activity (5) and the transcription of pro-inflammatory cytokines (6).

Biological functions for IRE1α-XBP1 signaling

Multiple groups have identified key roles for IRE1α-XBP1 signaling in a number of organs and cell types through the use of conditional mouse models. Germline *Xbp1* deletion is embryonic lethal due to fetal liver failure (7). If this is rescued with a liver-specific *Xbp1* transgene, the mice die shortly after birth due to insufficient exocrine pancreas function (8). However, selective deletion of *Xbp1* or *Ern1* in the liver of adult mice results in marked reduction in serum triglyceride and cholesterol levels (4, 9). Selective deletion of *Xbp1* in pancreatic β cells results in mild hyperglycemia and glucose intolerance (10). In the hematopoietic system, XBP1 is a key, cell-intrinsic requirement for plasma cell (11) and eosinophil differentiation (12), and mice with dendritic cell-specific *Xbp1* deletion exhibit reductions in splenic CD8α dendritic cells (13). Furthermore, XBP1 optimizes TLR-driven pro-inflammatory cytokine production in macrophages (6). Conditional deletion of *Xbp1* in the intestinal epithelium triggers Paneth cell death and colitic lesions resembling inflammatory bowel disease (14). However, this pathology is significantly attenuated in conditional *Ern1* knockout animals, suggesting that IRE1α hyperactivation leading to RIDD, which can occur after selective deletion of *Xbp1*, may be involved in exacerbating this inflammatory phenotype (15). Conditional deletion of *Xbp1* in the brain is neuroprotective in mouse models of Huntington’s disease (16) and ALS (17), while XBP1-mediated control of hexosamine biosynthesis in cardiomyocytes is cardioprotective in models of ischemia-reperfusion (18). Finally, animals lacking *Ern1* in all tissues except the placenta were viable and generally healthy, but displayed modest hyperglycemia and a reduction in serum antibody levels as predicted (19). The IRE1α-XBP1 signaling pathway therefore has a number of important physiological functions spanning multiple organ systems.

Cancer cell-intrinsic roles of IRE1α-XBP1 signaling

Malignant cells manage to survive under hostile conditions such as hypoxia and nutrient starvation via sustained activation of the IRE1α-XBP1 branch of the ER stress response (3, 20). Indeed, *XBP1* expression is increased in breast cancer cells resistant to anti-estrogen therapy (21) and high levels of *Xbp1s* transcripts are significantly associated with poor outcomes in endocrine-treated breast tumors (22). In addition, it was recently demonstrated *in vivo* that XBP1 drives triple negative breast cancer (TNBC) progression by cooperating with HIF1α to support tumor-initiating cell function and metastatic capacity of cancer cells under harsh environmental conditions (3). Therapeutic silencing of XBP1 in TNBC cells led to suppression of tumor initiation, progression, metastasis and recurrence, and high expression of XBP1-dependent gene signatures was found to be associated with worse prognosis in TNBC patients (3). XBP1 has also been demonstrated to drive the pathogenesis of multiple myeloma (23), and has been implicated in cancer cell de-differentiation, susceptibility to oncovirus infection and the epithelial-to-mesenchymal transition (24). Seminal work by the group of C.C. Andrew Hu also demonstrated constitutive IRE1α-XBP1 activation in murine chronic lymphocytic leukemia (CLL) cells, which promoted their pathogenesis *in vivo*. Accordingly, targeting IRE1α signaling *in vivo* with the selective small molecule endoribonuclease inhibitor B-I09 showed significant therapeutic effects, especially when used in combination with targeted anti-leukemic agents such as ibrutinib (25). In a xenograft model of human glioma, inhibiting IRE1α function by overexpressing a dominant negative variant significantly increased overall survival by decreasing tumor growth rate and angiogenesis (26). Furthermore, recent *in vivo* studies have also indicated that IRE1α-XBP1 signaling supports the aggressiveness of pancreatic cancer cells, and abrogating IRE1α activity using a small molecule inhibitor induced apoptosis and consequently delayed pancreatic tumor growth in xenograft models (27). Increasing evidence hence demonstrates that sustained IRE1α-XBP1 activation operates directly in cancer cells to promote tumor growth and metastasis *in vivo* in a variety of aggressive cancer types, many of which currently lack targeted therapies.

Immune cell dysfunction driven by abnormal IRE1α-XBP1 signaling

While IRE1α-XBP1 signaling has been shown to positively influence the growth and survival of malignant cells, the role of this cellular pathway in shaping the cancer immunoenvironment and the anti-tumor immune response had not been explored. Aggressive cancers recruit a broad collection of immune cells and effectively manipulate their intrinsic protective activity as a fundamental pro-tumoral mechanism. This process is epitomized by ovarian carcinoma, a highly immunosuppressive and lethal cancer that exquisitely controls normal dendritic cell (DC) functions in order to abrogate the generation of protective T cell-based responses (28). We hypothesized that common adverse conditions in the ovarian cancer microenvironment that induce protein misfolding (e.g. hypoxia, nutrient deprivation and/or oxidative stress) could trigger ER stress and robust activation of the IRE1α-XBP1 pathway in tumor-associated DCs (tDCs), a process that might influence their normal activity. Unlike DCs in non-tumor sites, DCs residing in human and mouse ovarian cancers exhibited robust and sustained IRE1α-XBP1 activation and concomitant overexpression of XBP1-dependent genes involved in the ER stress response (13). Mechanistically, high levels of reactive oxygen species (ROS) in tDCs promoted

intracellular lipid peroxidation and subsequent generation of reactive byproducts such as 4-hydroxynonenal (4-HNE), which induced ER stress by directly modifying critical ER-resident proteins and chaperones (13) (Figure 1). Treatment with antioxidants or pharmacological agents that efficiently sequester lipid peroxidation byproducts therefore prevented the induction of ER stress and IRE1α-XBP1 activation in DCs exposed to tumor-derived factors like those commonly present in malignant ovarian cancer ascites (13). We are currently defining the molecular mechanisms by which the tumor microenvironment fuels ROS accumulation and lipid peroxidation in tDC. Interestingly, lipid peroxidation byproducts have also been shown to promote vascular inflammation and atherogenesis by triggering ER stress in endothelial cells (29). Most importantly, ovarian cancer-bearing mice selectively lacking XBP1 in DCs demonstrated delayed progression of primary and metastatic ovarian tumors in three distinct preclinical models of disease (13). These effects correlated with enhanced intra-tumoral infiltration of activated, antigen-experienced T cells producing IFN-γ *in situ* (13), suggesting that tDC devoid of XBP1 were immunocompetent, rather than immunosuppressive. Global transcriptional profiling of tDCs revealed that constitutively active XBP1 not only promoted the expression of canonical XBP1-target genes involved in the ER stress response, but also induced a robust triglyceride biosynthetic program leading to abnormal lipid accumulation (Figure 1) (13). Interestingly, XBP1 had previously been demonstrated to drive hepatic lipogenesis by inducing the expression of key lipid biosynthetic genes (4). Seminal studies by the group of D. Gabrilovich had also uncovered that a major mechanism contributing to DC malfunction in cancer is indeed abnormal intracellular lipid accumulation. This dyslipidemia was shown to inhibit the efficient loading of antigenic peptides onto MHC-I molecules, thereby impairing optimal antigen cross-presentation to T cells by DCs (30). Consistent with this concept, XBP1-deficient tDC unable to accumulate intracellular lipid droplets demonstrated enhanced capacity to support T cell function both *in vitro* and *in vivo*, and memory (tumor-reactive) T cells generated in ovarian cancer-bearing mice selectively lacking XBP1 in DC demonstrated enhanced anti-tumor capacity when adoptively transferred into wild-type ovarian cancer hosts (13). We are currently exploring additional (lipid metabolism-independent) mechanisms by which sustained IRE1α-XBP1 activation promotes DC dysfunction in the tumor microenvironment.

Depleting or “licensing” tumor-associated myeloid cells *in vivo* has been widely used to restrain the optimal progression of several cancer types, but the precise microenvironmental conditions and molecular pathways that tumors exploit in these immune cells to co-opt their otherwise protective activity remain poorly understood. Our study provided the first evidence of a lethal cancer capable of co-opting IRE1α-XBP1 function in DCs of the tumor microenvironment as a strategy to evade immune control. This process may also orchestrate tolerance and immunosuppression in other lethal solid tumors that commonly rely on infiltrating innate immune cells to promote malignant progression. Future studies therefore aim at defining whether other cell types in the ovarian cancer immunoenvironment exhibit detrimental ER stress responses, and whether additional tumor types also rely on IRE1α-XBP1 signaling as a major immunosuppressive mechanism.

CLINICAL-TRANSLATIONAL ADVANCES

Small molecule inhibitors

Given that IRE1α-XBP1 signaling sustains both cancer cell-extrinsic immunosuppression and cancer cell-intrinsic growth and metastasis, there is significant interest in developing targeted therapies against this UPR pathway. While technical limitations preclude the development of direct small molecule XBP1 inhibitors, the formation of the active, spliced *Xbp1* variant can be readily targeted via its dependency on IRE1α. The dual enzyme IRE1α is amenable to small molecule targeting, and multiple inhibitor classes have been identified from various independent small molecule screens. Several crystal structures of IRE1α in complex with either kinase inhibitors or hydroxyl-aryl-aldehyde endoribonuclease inhibitors have been published (31, 32), enabling rational development of novel IRE1α inhibitors.

Small molecule IRE1α inhibitors can be grouped into three main categories based on their structures and mode of action. The first group consists of inhibitors with indirect or unknown mechanisms of action, and include irestatin, trierixin (33) and quinotrierixin (34). These compounds were each identified by screening small molecule libraries against human cell lines expressing IRE1α endoribonuclease-driven luciferase reporter plasmids in the presence of chemical ER stressors such as thapsigargin or tunicamycin. In these reporter systems, the firefly luciferase cDNA is fused out of frame to a fragment of human XBP1 cDNA bearing IRE1α splicing recognition sites, and is only translated in-frame upon IRE1α-mediated RNA splicing. The IRE1α inhibitory capacity for each inhibitor was subsequently confirmed with luciferase-independent, PCR-based methods in human cell lines. However, the mechanisms underlying inhibitor activity remain poorly defined, and it is unclear whether these compounds specifically target IRE1α or whether they interfere with UPR activation more generally, as has been suggested for quinotrierixin (34).

The second and largest group of inhibitors is comprised of direct IRE1α endoribonuclease inhibitors. Some of these compounds were identified in high-throughput screens against the endoribonuclease activity of the purified IRE1α cytoplasmic domain, while others were developed during optimization efforts on pre-existing leads. Most of these compounds, including 3-ethoxy-5,6-dibromosalicylaldehyde (35), 4μ8C (36), MKC-3946 (37), and B-I09 (25), are salicylaldehyde and coumarin derivatives which generally share a core hydroxyl-aryl-aldehyde (HAA) structure. Crystallographic analyses have demonstrated that these HAA inhibitors bind covalently to lysine K907, which resides in a shallow, solvent-exposed pocket on the IRE1α endoribonuclease domain (31). However, this HAA motif is not an absolute structural requirement, as both STF-083010 (38) the nucleoside-type antibiotic analogue toyocamycin can also directly block the IRE1α endoribonuclease (39). All direct IRE1α endonuclease inhibitors dose-dependently reduced *Xbp1* splicing *in vitro* in human cell lines without affecting IRE1 phosphorylation or signaling from the PERK and ATF6. Importantly, STF-083010 (38), MKC-3946 (37) and toyocamycin (39) demonstrated efficacy against multiple myeloma both *in vitro* and in xenograft survival studies, and B-I09 reduced tumor burden in a genetic mouse model of CLL driven by the Eμ-TCL1 transgene (25). Furthermore, daily intraperitoneal administration of 4μ8C significantly reduced pathological joint swelling in the KBxN serum transfer murine model of rheumatoid arthritis

(40). Cumulatively, these reports validate IRE1α as an attractive clinical target and indicate that the endoribonuclease domain is chemically tractable.

The final group of small molecule IRE1α inhibitors is kinase inhibitors, which act allosterically to disrupt endoribonuclease function. Compared with the extensive and rapidly expanding collection of endoribonuclease inhibitors, IRE1α kinase inhibitors are considerably less well developed despite their significant therapeutic potential. This disparity may be due in part to nuances in how the IRE1α endoribonuclease domain responds to different classes of kinase inhibitors. When the IRE1α kinase DFG loop shifts into a "DFG-in" conformation, a structure stabilized by certain type I kinase inhibitors like sunitinib and a novel compound known as "Compound 3", the endoribonuclease domain cleaves the *Xbp1* mRNA in the absence of IRE1α autophosphorylation (41). However, upon adopting a "DFG-out" conformation, which can be enforced with certain type II kinase inhibitors such as KIRA6 and AD60 (42), both the kinase domain and the endoribonuclease domain are rendered inert (43). Interestingly, in male *Ins2*$^{+/Akita}$ mice, which express a mutated pro-insulin that causes chronic ER stress in pancreatic β-cells, twice daily administration of KIRA6 reduced plasma glucose levels and improved glucose tolerance test outcomes (43). Furthermore, intravitreal KIRA6 injection in the P23H transgenic rat model of retinitis pigmentosa preserved photoreceptor viability and function (43). These *in vivo* data are consistent with accumulating evidence suggesting that protein misfolding and ER stress may be linked to both metabolic dysfunction and retinal degeneration. Though kinase domains are highly structurally conserved, extremely selective IRE1α kinase inhibitors can be generated, as illustrated by the recently reported "Compound 18" and GSK2850163 (32, 44). However, the *in vivo* effects of these selective compounds were not reported. Type II kinase inhibition, but not type I kinase inhibition, therefore represents a second pharmacologically tractable strategy for globally blocking IRE1α endoribonuclease activity in the tumor microenvironment.

Immune cell-specific approaches

Due to unique properties of the immune system, other small molecule-independent strategies could also be utilized to disable IRE1α-XBP1 signaling selectively in DCs of cancer hosts. First, DCs within malignant ovarian cancer ascites have exceptional phagocytic capacity, rendering them excellent targets for nanoparticle-mediated RNAi therapeutics (45). As ovarian cancer metastasis is generally confined within the peritoneal cavity, intraperitoneal administration of siRNA-loaded nanoparticles targeting *Ern1* or *Xbp1* represents a novel and feasible immuno-oncology strategy. In animal models of established metastatic ovarian cancer, silencing *Xbp1* expression using this approach rendered tDCs highly immunostimulatory and significantly extended host survival by stimulating T cell-mediated anti-tumor immunity (13).

As a second strategy, the genes encoding IRE1α or XBP1 could be ablated to enhance the efficacy of autologous DC adoptive transfer strategies. Despite the modest successes of adoptive DC therapy, ovarian cancer patients were refractory to similar tumor antigen-pulsed adoptive DC treatments (46). Genome editing technologies such as CRISPR/CAS9, zinc finger nucleases, or TALENs (47) should enable precise and efficient mutation of *XBP1* or

ERN1 in DCs prior to adoptive transfer, thereby protecting these transplanted DCs from the suppressive effects of aberrant ER stress responses induced by the tumor microenvironment. In proof-of-concept experiments, we demonstrated that transferring *Xbp1*-deficient BMDCs into mice with established primary ovarian cancer significantly delayed tumor progression compared with infusion of wild type BMDCs (13). Strikingly, transplanted *Xbp1*-deficient DCs were dominantly immunostimulatory over the endogenous (wild type) regulatory DCs residing in the tumor microenvironment. Hence, cutting-edge genetic methods for targeting IRE1α-XBP1 signaling would likely enhance the efficacy of current adoptive DC therapies in ovarian cancer.

To conclude, the IRE1α-XBP1 branch of the ER stress response is a novel and well-characterized pathway with significant therapeutic relevance in a variety of human cancers. This molecular pathway controls unique biological processes in the cancer cell and in tumor-infiltrating immune cells to ultimately promote tumor progression. While IRE1α-XBP1 signaling can be targeted through a variety of classical and non-classical methods (Figure 2), potent small molecule inhibitors represent an attractive strategy to simultaneously disable this pro-tumoral pathway in the cancer cell and in the innate immune system.

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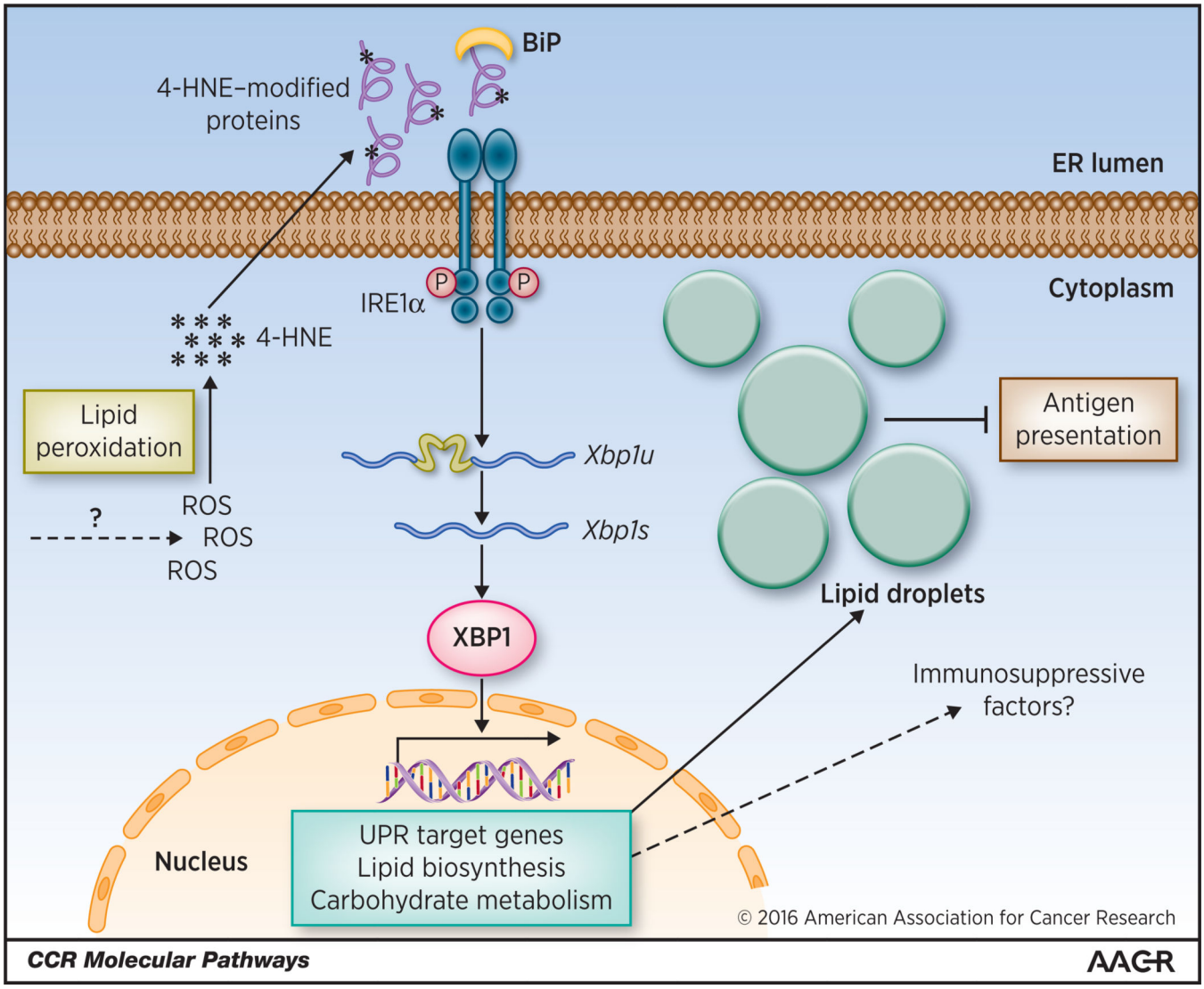

Figure 1. Activation and function of IRE1α-XBP1 in ovarian cancer-resident DCs

High levels of ROS in tDCs promote intracellular lipid peroxidation and generation of reactive byproducts like 4-HNE. This diffusible aldehyde modifies ER resident proteins and triggers the ER stress response, thus inducing activation of IRE1α and subsequent cleavage of the *Xbp1u* mRNA to generate the spliced *Xbp1s* form. Constitutively active XBP1 promotes expression of genes involved in the UPR, carbohydrate metabolism and lipid biosynthesis. Aberrant lipid accumulation inhibits the capacity of tDCs to present local tumor antigens to infiltrating T cells.

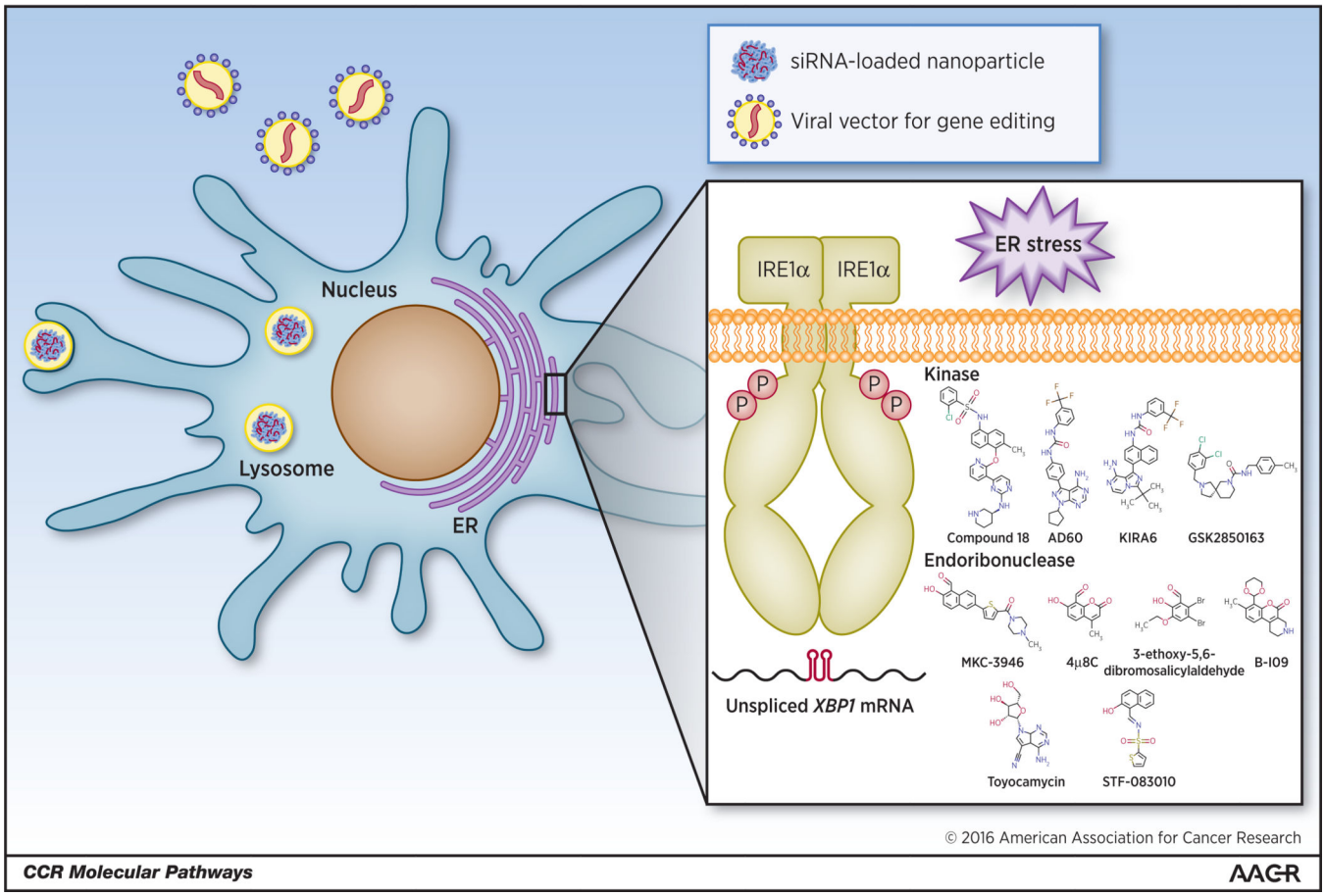

Figure 2. Potential therapeutic strategies for targeting IRE1α-XBP1 signaling in cancer-associated DCs

The IRE1α kinase and endoribonuclease domains can be individually blocked with small molecules. Additionally, the constitutive activation of either XBP1 or IRE1α can be reduced using nanoparticles encapsulating siRNAs. In terms of DC-based vaccines or autologous DC transfer, IRE1α or XBP1 could be selectively ablated *ex vivo* through the use of virally-delivered DNA editing technologies such as zinc finger nucleases, TALENs, and CRISPR/CAS9. Genome edited DCs could then be transplanted back into patients to enhance standard therapeutic regimes.