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BRIEF REPORT

Surface-exposed and soluble calreticulin: conflicting biomarkers for cancer prognosis

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ABSTRACT

Increased exposure of calreticulin (CALR) on malignant cells is associated with therapy-relevant adaptive immune responses and superior therapeutic outcome in solid tumors and haemato-oncological diseases, because surface-exposed CALR acts as an 'eat-me' signal facilitating the phagocytosis of stressed and dying cancer cells by immature dendritic cells, thus favoring antitumor immune responses. On the contrary, mutations of the *CALR* gene that cause the omission of the C-terminal KDEL endoplasmic reticulum retention motif from CALR protein, resulting in its secretion from cells, act as oncogenic drivers in myeloproliferative neoplasms via the autocrine activation of the thrombopoietin receptor. We recently showed that soluble CALR inhibited the phagocytosis of cancer cells by dendritic cells, thus dampening anticancer immune responses. Furthermore, systemic elevations of soluble CALR that is secreted from tumors or that is artificially supplied by injection of the recombinant protein decreased the efficacy of immunotherapy. Thus, depending on its location, CALR can have immunostimulatory or immunosuppressive functions.

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Calreticulin (CALR) is an endoplasmic reticulum (ER)-resident chaperone that mediates protein (re)folding and facilitates cellular Ca^{2+} storage and homeostasis. The full-body knockout of CALR is embryonically lethal, emphasizing its crucial function for the regulation of cellular homeostasis.[1] The CALR protein consists of three structurally and functionally distinct domains, the N-terminus that contains the ER-signal sequence, followed by the proline-rich *P*-domain harboring the chaperone function and the C-terminus with the KDEL tetrapeptide motif for ER retention.[2] The compartmental targeting (N-terminus) and the KDEL-mediated retention (C-terminus) together dictate the confinement of CALR to the ER lumen in cellular homeostasis. However, in response to specific stress signals impacting the ER, CALR can translocate to distinct cellular compartments, thereby modulating a broad variety of physiological processes including cell adhesion and migration as well as the phagocytosis of apoptotic cells, thus impacting on diverse processes such as wound healing and immune responses.[3]

In specific circumstances, for instance in response to chemotherapy with anthracyclines or oxaliplatin (which are chemotherapeutics inducing "immunogenic cell death", ICD), cancer cells experience a partial ER stress response, that involves the phosphorylation of eukaryotic translation initiation factor 2α (EIF2A, better known as eIF2α), mostly by eukaryotic EIF2A kinase 3 (EIF2AK3, better known as PERK) and in some instances by EIF2AK4 (better known as GCN2), but does not comprise the activation of other facets of the unfolded stress response involving ATF4, ATF6 or XBP1. It is in this particular context of a focused ER stress response that CALR can translocate to the surface of the cytoplasmic membrane. Once exposed at the outer leaflet of the cellular membrane, CALR serves as a danger associated molecular pattern (DAMP) for the recognition of stressed and dying cells by the innate immune system, in particular immature dendritic cells (DCs), which are the most important antigen-presenting cells.[4-8] Indeed, eIF2α phosphorylation appears to be required for ICD, as this has been shown for both anticancer chemotherapy and radiotherapy.[7,9-12] In this settings, surface-exposed CALR acts as *de novo* uptake signal via the ligation of pattern recognition receptors (PRRs) such as low density lipoprotein receptor-related protein 1 (LRP1, better known as CD91) on DCs, thus facilitating the engulfment of tumor-associated antigens expressed by malignant cells and their MHC class I-restricted cross-presentation to $CD8^+$ T lymphocytes.[13,14] (Figure 1) As such, CALR exposure constitutes a central adjuvant component of ICD, that ultimately facilitates the induction of cytotoxic T cell (CTL)-mediated anticancer immune responses.[9] Elevated CALR exposure on the outer leaflet of cancer cells has been linked with anti-cancer immunity and superior therapeutic outcome in patients with non-small cell lung carcinoma (NSCLC), colorectal carcinoma (CRC), acute myeloid leukemia (AML), ovarian cancer and high-grade serous carcinomas (HGSCs).[15-19] (Table 1) In this setting, elevated levels of CALR mRNA and protein expression at the site of the tumor correlate with beneficial disease outcome. A recent work from Truxova and collaborators discussed the ability of CALR exposed by malignant blasts from

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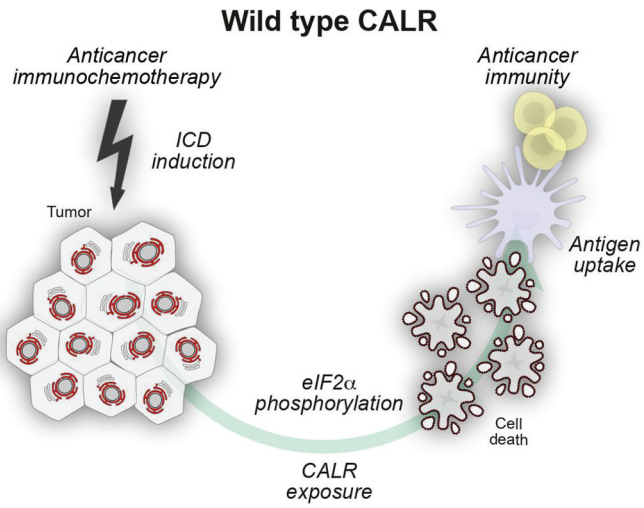

Figure 1. Surface-exposed calreticulin serves as an uptake signal for dendritic cells. Certain anticancer regimens induce T cell-dependent adaptive anticancer immunity via the initiation of immunogenic cell death (ICD). One of the apical hallmarks of ICD is a partial endoplasmic reticulum (ER) stress response that leads to the phosphorylation of eIF2α in the absence of other manifestations of the unfolded protein response. The resultant exposure of calreticulin (CALR) on the surface of dying cells facilitates their recognition by dendritic cells (DC) and thus enables tumor-associated antigen transfer culminating in adaptive anticancer immunity.

acute myeloid leukemia patients to trigger an IL-15-mediated trans-presentation and activation of natural killer cells further corroborating its importance for anti-cancer immunity.[21] On the contrary, the expression of the surface protein CD47 on cancer cells can transmit an inhibitory "don't eat me" signal upon the ligation with its receptor signal regulatory protein α (SIRPα), primarily expressed on phagocytic cells such as DCs. Thus the overabundance of CD47 may potentially play an important role in tumor cell evasion.[22-27]

Soluble CALR can be passively released or actively secreted from cells, as this occurs in the context of massive cell death upon anticancer chemotherapy. Moreover, CALR can be secreted by macrophages stimulated by toll-like receptor (TLR) agonists and then contributes to the recognition and phagocytosis of adjacent tumor cells.[32] In addition, extracellular soluble CALR stimulates wound healing, as well as neoangiogenesis.[31]

Exon 9 mutations of *CALR* have been identified in up to 30% of patients affected by myeloproliferative neoplasms (MPNs) such as essential thrombocythemia (ET) and myelofibrosis (MF).[33,34] The most recurrent mutations typically manifest as either a 52 base pair deletion of residues 1092 to 1142 (*CALRdel52*) or a 5 base pair insertion between residues 1154 and 1155 (*CALRins5*). Both mutations lead to an alternative open reading frame, resulting in similar changes in the C-terminal amino acid sequence of the protein that becomes positively charged and loses the KDEL ER retention signal.[35] Consequently, mutant CALR protein fails to be detected by KDEL retention receptors and thus enters the conventional protein secretion pathway and is released via Golgi-mediated exocytosis.[30,36,37] Secreted CALR mutants bind (via their lectin binding sites) to the extracellular domain of the thrombopoietin receptor (MPL) in a cell autonomously or paracrine fashion thus leading to a downstream activation of the Janus kinase 2 (JAK2) and signal transducer and activator of transcription (STAT) proteins STAT1, STAT3 and STAT5.[38-41] Introduction of analogous CALR mutations into mice recapitulates the ET-like disease and its progression to myelofibrosis.[41-43] Thus, CALR mutants act as oncogenic driver of MPN.[39,41,44,45] Moreover, patients with MPN-associated CALR mutations exhibit an increase in myeloid derived suppressor cells (MDSC) and immunosuppressive B cells, suggesting that mutated CALR may subvert immune responses.[44,46]

Table 1. CALR as a biomarker in human cancer.

Cancer type	Therapeutic outcome	Remarks	Ref.
Acute myeloid leukemia (AML)	CALR exposure is associated with improved relapse-free survival (RFS) and correlates with superior overall survival (OS).	CALR exposure correlates with an increase in effector memory $CD4^+$ and $CD8^+$ T cells specific for AML antigens	19,20
Breast cancer (BC)	Not determined	CALR mRNA expression correlates with tumor immune infiltration	17
Colorectal cancer (CRC)	CALR expression is associated with increased 5-year survival rate	CALR expression is associated with the infiltration of tumors by $CD45RO^+$ cells	16
Non-small cell lung cancer (NSCLC)	CALR expression correlates with increased OS	CALR expression correlates with increased infiltration of tumors by dendritic cells and $CD8^+$ T cells	17,18
Ovarian cancer	CALR expression correlates with increased RFS and OS	CALR exposure correlates with TH1 polarization and cytotoxic activity.	15,17

Table 2. Secreted CALR as a biomarker in myelofibrosis and solid tumors.

Cancer type	CALR effects	Outcome	Ref.
CALR mutated-MPN (ET and MF)	Activation of JAK2/STAT pathway through MPL binding	Megakaryocytic hyperplasia	28
MPN (ET and MF)	Reduced phagocytosis	Immunosuppressive effects	29
MPN (ET and MF)	Increased cytokine production from normal monocytes	Inflammation	30
Solid cancers with $CALR^{E405*}$ or $CALR^{X352}$	Suppressing the antitumor immune response	Immunosuppressive effects	29
Human colon carcinoma and Burkitt lymphoma	Inhibition of endothelial cell growth by vasostatin	Suppression of neovascularization; reduced tumor growth	31

Abbreviations: Essential thrombocythemia (ET), Janus kinase (JAK), myelofibrosis (MF), myeloproliferative leukemia protein (MPL), myeloproliferative neoplasm (MPN), signal transducer and activator of transcription (STAT)

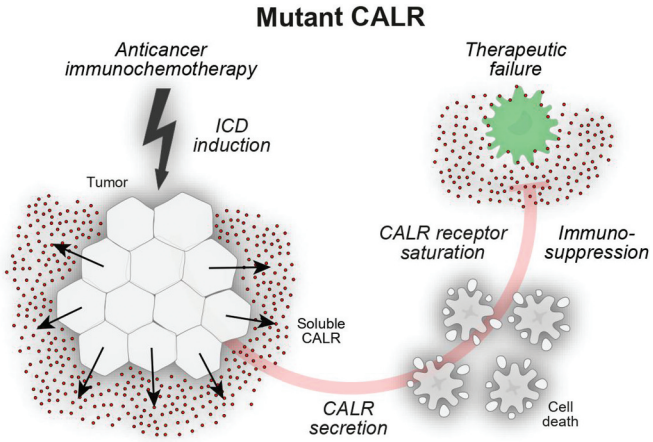

Figure 2. Secreted calreticulin negatively impacts on phagocytosis. Calreticulin (CALR) mutants that affect the C-terminal endoplasmic reticulum (ER)-retention signal KDEL can enter the canonical protein secretion pathway. Soluble CALR protein is secreted via Golgi-dependent exocytosis and ligates surface receptors on antigen presenting cells. In this setting, soluble CALR acts as a decoy that triggers receptor saturation and inhibits the dendritic cell (DC)-mediated phagocytosis of stressed and dying cancer cells, thus blunting adaptive immunity to tumor-associated antigens.

Recently, we showed that exon 9-mutated CALR is not only secreted from cells via Golgi-mediated protein secretion but also acquires a novel immunosuppressive function, once it accumulates in the extracellular space. Indeed, soluble CALR acts as a decoy, preventing the phagocytosis of CALR-exposing cells by DC *in vitro*, as well as *in vivo*, in mice (Figure 2). The release of soluble CALR from tumors mediated robust immunosuppressive effects and abolished therapeutic responses to immunogenic chemotherapy (with anthracyclines or oxaliplatin) as well as immune checkpoint blockade targeting the PD-1/PD-L1 interaction.[29]

In the context of MPNs, soluble CALR provides both an autocrine signal for the activation of an oncogenic driver fostering disease progression and subverts phagocytosis of malignant cells thus jeopardizing anticancer immune responses. This might explain the precocious initiation of MPN associated with CALR mutants, occurring some 10 years earlier than in MPN associated with the JAK2V617 F mutation.[44] This correlates with the fact that *CALR* mutations are present in all hematopoietic cells (both myeloid and lymphoid) and give a high clonal dominance to the hematopoietic stem compartment compared to *JAK2*V617 F in ET.[43,47] (Table 2) Preclinical experiments suggest that mutations leading to the release of CALR in MPNs would interfere with therapeutic measure designed to (re)establish an anticancer immune response, a conjecture that must be validated at the clinical level. Of note, we found that a small percentage (<1%) of human carcinomas also exhibited CALR mutations in exon 9 and that such mutations, which are different from the MPN-associated ones, also led to the secretion of CALR protein, as well as to the subversion of therapeutic responses to immunogenic chemotherapy or PD-1 blockade in preclinical experiments. It will be important to investigate the clinical impact of such CALR mutations for patient prognosis.

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Disclosure of potential conflicts of interest

OK and GK are scientific co-founders of Samsara Therapeutics.

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