Cell Research

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REVIEW ARTICLE

Calreticulin and cancer

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Calreticulin (CALR) is an endoplasmic reticulum (ER)-resident protein involved in a spectrum of cellular processes. In healthy cells, CALR operates as a chaperone and Ca^{2+} buffer to assist correct protein folding within the ER. Besides favoring the maintenance of cellular proteostasis, these cell-intrinsic CALR functions support Ca^{2+}-dependent processes, such as adhesion and integrin signaling, and ensure normal antigen presentation on MHC Class I molecules. Moreover, cancer cells succumbing to immunogenic cell death (ICD) expose CALR on their surface, which promotes the uptake of cell corpses by professional phagocytes and ultimately supports the initiation of anticancer immunity. Thus, loss-of-function *CALR* mutations promote oncogenesis not only as they impair cellular homeostasis in healthy cells, but also as they compromise natural and therapy-driven immunosurveillance. However, the prognostic impact of total or membrane-exposed CALR levels appears to vary considerably with cancer type. For instance, while genetic *CALR* defects promote pre-neoplastic myeloproliferation, patients with myeloproliferative neoplasms bearing *CALR* mutations often experience improved overall survival as compared to patients bearing wild-type *CALR*. Here, we discuss the context-dependent impact of CALR on malignant transformation, tumor progression and response to cancer therapy.

Cell Research (2021) 31:5–16; https://doi.org/10.1038/s41422-020-0383-9

INTRODUCTION

According to current models, oncogenesis involves two main, highly-interconnected components: (1) the accumulation of genetic and/or epigenetic defects that endow (initially) normal cells with an increased proliferative potential, an accrued resistance to cell death, the ability to drive neo-angiogenesis, as well as the capacity to disseminate to form metastases,[1] and (2) the escape from local and systemic control by the host immune system (immunoevasion).[2,3] Thus, malignant transformation (i.e., the process through which a healthy cell becomes a neoplastic cell precursor) and tumor progression (i.e., the proliferation of such precursor coupled with the acquisition of increasingly malignant features), as well as the response of established tumors to therapy, occur in the context of a bidirectional interaction between (pre-)malignant cells and their host that ultimately dictates clinical outcome.[4–7] In this context, alterations that simultaneously endow cancer cells with an intrinsic proliferative advantage and limit the ability of the immune system to recognize and eliminate them (Box 1) are expected to be potent drivers of oncogenesis.

Calreticulin (CALR) is a Ca^{2+}-binding endoplasmic reticulum (ER) protein that aids the folding of proteins destined to secretion and insertion in the plasma membrane, de facto providing a major contribution to the maintenance of cellular homeostasis when unfolded proteins accumulate within the ER (e.g., in the context of viral infection).[8–10] Moreover, CALR mechanistically contributes to the initiation of adaptive anticancer immunity in the context of immunogenic cell death (ICD), a functional variant of regulated cell death (RCD) that is sufficient to elicit an antigen-specific immune response in immunocompetent, syngeneic hosts (provided that dying cells express antigens not covered by central or peripheral tolerance).[11–13] In particular, CALR exposed on the surface of cancer cells undergoing ICD mediates robust pro-phagocytic effects, hence facilitating the uptake of dying cells or their corpses by antigen-presenting cells (APCs), in particular immature dendritic cells (DCs), that migrate to lymph nodes to cross-prime tumor-specific naïve $CD8^+$ T cells.[11,12]

Thus, wild-type CALR expressed at physiological levels operates at the interface between the preservation of cellular homeostasis and the initiation of an immune response that eradicates cells experiencing damage beyond recovery in support of organismal fitness.[14,15] In line with this notion, CALR is mutated or down-regulated in a variety of neoplasms.[4,16,17] Here, we discuss the cell-intrinsic and immunological mechanisms whereby CALR influences malignant transformation, tumor progression and response to therapy.

CALR IN CELLULAR HOMEOSTASIS

CALR is a highly conserved Ca^{2+}-binding chaperone of 417 amino acids (MW: 46 kDa) that is predominantly localized in the

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Received: 23 April 2020 Accepted: 13 July 2020
Published online: 30 July 2020

Box 1 Cancer immunosurveillance at a glance

For a long time, cancer was considered to originate solely from genetic or epigenetic defects affecting one (or a few) neoplastic cell precursor(s) that progressively acquire(s) the ability to proliferate relentlessly, resist cell death, promote the formations of new vessels and invade local as well as distant sites to generate metastases.[1] However, pioneer work from the late 1990s and early 2000s suggested that the host immune system has a key role in the control of emerging and progressing tumors.[174–176] Since then, an abundant literature has accumulated in support of the notion that the immune system eliminates (pre) neoplastic cells as they form, but the latter — at least in some cases — can acquire additional (epi)genetic alterations that result first in an equilibrium phase, and then in overt immunoevasion coupled to local and distant progression.[177] Importantly, the pressure imposed on developing tumors by the host immune system operates as an evolutionary bottleneck, de facto limiting the immunogenicity (or accruing the immunosuppressive functions) of cancer cells that successfully evade immunosurveillance.[3,178] According to current models, cancer immunosurveillance is mainly mediated by a specific subset of classical dendritic cells known as cDC1 cells,[179–181] type I helper (T_H1) $CD4^+$ cells, $CD8^+$ cytotoxic T lymphocytes (CTLs)[182–184] and (at least in some oncological settings) natural killer (NK) cells.[185–187] In particular, while cDC1 cells, T_H1 $CD4^+$ cells, and $CD8^+$ CTLs support the elimination of cancer cells expressing antigens that are not covered by thymic or peripheral tolerance (adaptive immunosurveillance),[188–191] NK cells preferentially target neoplastic precursors or metastatic cancer cells that express NK cell-activating ligands on their surface in response to microenvironmental stress.[185,186,192,193] The role of other immune cells in the control of oncogenesis and tumor progression remains to be clarified. Indeed, granulocytes, macrophages and B cells all appear to resemble DCs as they can exist in an ample spectrum of phenotypic and functional states spanning from purely immunostimulatory and anti-cancer to purely immunosuppressive and pro-cancer.[194–201]

ER lumen.[18] CALR consists of three distinct domains: (1) an N-terminal lectin-like globular domain that is responsible for the interaction of CALR with α integrins and contains a steroid receptor-like DNA-binding site involved in chaperone functions; (2) a central proline-rich (P) domain with high-affinity and low-capacity for Ca^{2+} binding that participates in CALR chaperone activity; and (3) a highly acidic C-terminal region with low-affinity and high-capacity for Ca^{2+} binding that is involved in CALR Ca^{2+}-buffering functions, followed by a C-terminal KDEL domain that ensures CALR retrieval from the Golgi apparatus to the ER[18,19] (Fig. 1a).

As a reticular protein, CALR is involved in a quality control system for newly synthesized proteins and glycoproteins that relies on multiple additional chaperones, including (but not limited to) calnexin (CANX), heat shock protein family A (Hsp70) member 5 (HSPA5, also known as BiP or GRP78), heat shock protein 90 beta family member 1 (HSP90B1, also known as GRP94), protein disulfide isomerase family A member 3 (PDIA3).[20] Globally, such a system (which is commonly known as the CALR/CANX cycle) prevents the premature export of misfolded proteins from the ER as it supports refolding[20] (Fig. 1a), hence occupying a central position in the homeostatic response to the accumulation of unfolded polypeptides.[9] Alongside, the Ca^{2+}-binding functions of CALR are central for physiological Ca^{2+} and integrin signaling in both excitable and non-excitable cells.[21–23] In particular, a cytosolic pool of CALR is required for integrin-dependent cell adhesion, reflecting the ability of CALR to reversibly bind the KxGFFFKR domain in the cytoplasmic tails of α integrins.[23,24] In line with the key role of CALR in whole-body physiology, $Calr^{-/-}$ mice die in utero at day 12.5–16.5 as they fail to absorb the umbilical hernia and manifest prominent cardiac alterations.[25] Interestingly, such developmental alterations seem to result from transcriptional defects caused by Ca^{2+}-related nuclear pore complex malfunction,[26] rather than from reduced contractility. Consistent with this interpretation, the adult heart expresses low CALR levels[25] and transgene-enforced CALR overexpression in the adult heart causes contractility issues that culminate with complete heart block and sudden death.[27] Moreover, the $Calr^{-/-}$ genotype imposes functional defects to a variety of cell types beyond cardiomyocytes,[28] such as T cells,[29] endothelial cells,[30] vascular smooth muscle cells,[31] fibroblasts,[32] and oocytes.[33]

Recurrent somatic mutations in *CALR* affecting the majority of patients with myeloproliferative neoplasms (MPNs) who do not bear mutations in Janus kinase 2 (*JAK2*) and MPL proto-oncogene, thrombopoietin receptor (*MPL*) were first documented in 2013.[34,35] Such *CALR* mutations most often consist of insertions and/or deletions in exon 9, resulting in a C-terminal domain that bears a novel, positively-charged amino acid sequence and lacks the KDEL domain (Fig. 1b), thus enabling mutant CALR to escape the ER and form stable complexes with MPL by interacting with glycans on MPL N117 residue.[36,37] These complexes are exposed on the surface of hematopoietic precursors through normal anterograde ER-to-Golgi transport, culminating with thrombopoietin (TPO)-independent MPL dimerization, consequent JAK2 and mitogen-activated protein kinase (MAPK) activation, and final signal transducer and activator of transcription (STAT) signaling.[36,38–40] Ultimately, this cascade drives the deregulated clonal expansion of hematopoietic stem cells and megakaryocytes that underlies MPNs, as documented in a variety of animal models.[41–43] In support of this hierarchical signal transduction model, the oncogenic potential of human *CALR* mutations is highly compromised upon the depletion of either MPL or JAK2, as well as in the presence of pharmacological JAK2 inhibitors.[44,45]

Preclinical data suggest that type I *CALR* mutations, which eliminate all negatively-charged amino acids from the CALR C-terminus (e.g., del52), appear to be superior to their type II counterparts, which only eliminate approximately half of such residues (e.g., ins5), at driving thrombocytosis progression to myelofibrosis,[40,46] an oncogenic function that strictly depends on the CALR N-terminal domain.[47] Consistent with this notion, patients with type I *CALR* mutations experience a more aggressive disease course with rapid progression to acute lymphoid leukemia (AML) as compared to individuals with type II mutations.[48] Of note, both type I and type II *CALR* mutations not only promote constitutive MPL signaling to support TPO-independent proliferation, but also compromise (at least to some degree) cellular responses to unfolded protein accumulation and oxidative stress,[49] thus promoting oncogenesis via accrued generation of reactive oxygen species (ROS) and consequent genomic instability.[50] Moreover, both type I and type II *CALR* mutations have recently been shown to cause TPO-independent Ca^{2+} fluxes in megakaryocytes,[51] thus promoting uncontrolled proliferation.[52] As type I mutations are expected to impair the Ca^{2+}-binding functions of CALR more than their type II counterparts, this latter mechanism may contribute (at least partially) to the differential disease course of patients affected by type I versus type II *CALR* mutations.

Taken together, these observations delineate a precise molecular pathway whereby genetic defects in *CALR* support malignant transformation in the hematopoietic system via trophic, cell-intrinsic mechanisms. The loss of wild-type CALR functions, however, is also expected to favor oncogenesis by compromising immunosurveillance, as discussed below.

CALR IN ANTIGEN PRESENTATION

CALR is an integral part of the so-called peptide-loading complex (PLC), a transient multicomponent complex that assembles at the membrane of the ER to ensure the proper loading of cellular antigens onto MHC Class I molecules.[53–55] Besides CALR, the PLC involves PDIA3, TAP-binding protein (TAPBP, also known as tapasin), transporter 1, ATP-binding cassette subfamily B member (TAP1) and transporter 2, ATP-binding cassette subfamily B member (TAP2), which collectively mediate: (1) the assembly of MHC Class I heavy chains with beta 2 microglobulin (B2M); (2) the ATP-dependent transportation of cytosolic peptides to the ER lumen; (3) the loading of such peptides on the antigen-binding

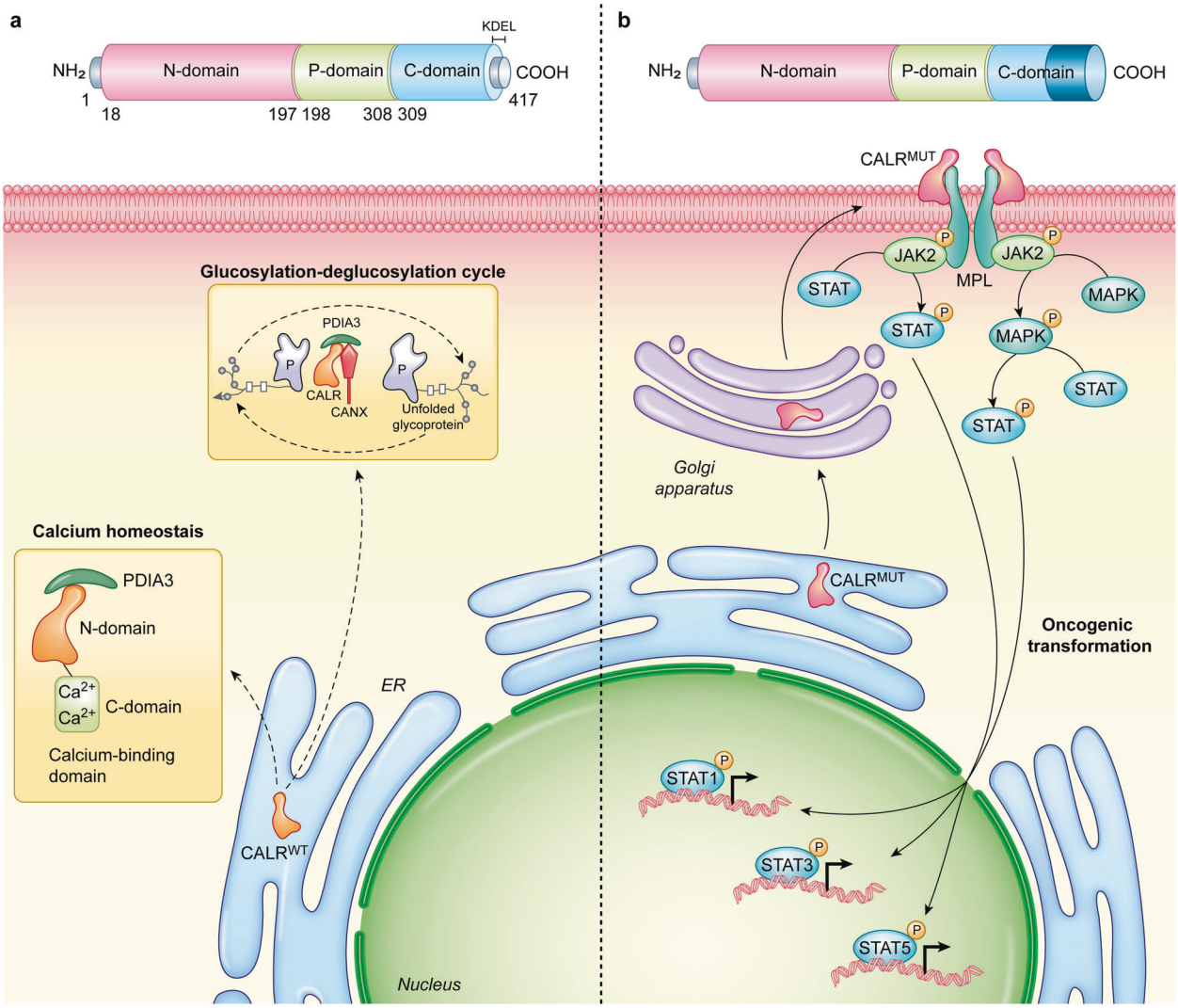

Fig. 1 Cell-intrinsic functions of wild-type and mutant calreticulin. a Wild-type calreticulin ($CALR^{WT}$) mediates key functions not only as it cooperates with CANX and PDIA3 in the control of protein folding within the ER, but also as it contributes to reticular Ca^{2+} buffering. **b** Cancer-associated *CALR* mutations compromise the capacity of mutant CALR ($CALR^{MUT}$) to be retained in the ER, resulting in anterograde CALR transport from the ER to the plasma membrane via the Golgi apparatus, constitutive association with MPL, and TPO-independent oncogenic signaling (at least in some cells) via JAK2 and MAPKs. P, phosphate.

pocket of MHC Class I molecules, and (4) the release of loaded MHC Class I for anterograde ER-to-Golgi transport and exposure on the plasma membrane.[56] CANX also participates in peptide loading by binding to (and hence stabilizing) MHC Class I heavy chains prior to their stabilizing interactions with B2M[56] (Fig. 2).

Specifically, CALR contributes to PLC functions by at least two different mechanisms.[57] First, by interacting with PDIA3 in a glycan-dependent manner, CALR preserves steady-state levels of TAPBP and MHC Class I heavy chains.[58,59] Second, CALR can retrieve suboptimally assembled MHC Class I molecules from post-ER compartments, notably the ER-Golgi intermediate compartment (ERGIC) and the *cis*-Golgi.[60] Thus, both mouse and human cells lacking CALR exhibit a major (50%–80%) reduction in peptide loading onto MHC Class I molecules and exposure of properly loaded MHC on the cells surface,[61] a defect that cannot be rescued by re-expression of CALR variants lacking the C-terminus.[62] Consistent with this, cancer-related CALR mutants are unable to support the activity of the PLC and hence are associated not only with constitutive MPL signaling (see above), but also with reduced antigen presentation on MHC Class I molecules,[63] de facto favoring immunoevasion upon loss of tumor antigenicity.[64] Further supporting this notion, even in the absence of oncogenic mutations, CALR levels are reduced in multiple solid tumors at advanced stage, generally correlating with reduced MHC Class I exposure on the cell surface and poor disease outcome.[65–67]

Apparently at odds with the above, at least some cancer-relevant mutations of *CALR* exon 9 have been shown to generate shared tumor neoantigens (TNAs) eliciting spontaneous immune responses in patients with MPNs.[68,69] Such responses, however, largely rely on antigen presentation by MHC Class II molecules and appear to involve a population of $CD4^+$ T cells with cytolytic functions,[68] explaining why they can arise in the context of impaired MHC Class I presentation. Of note, spontaneous CALR-targeting immune responses in MPN patients appear to be under strict regulation by cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death 1 (PDCD1, best known as PD-1) signaling.[70] Consistent with this, administration of the PD-1-targeting immune checkpoint blocker (ICB) pembrolizumab restored T cell immunity against mutant CALR in some patients with MPN.[70] Notably, $CD8^+$ T cell responses were also detected in this setting,[70] potentially reflecting the ability of interferon gamma

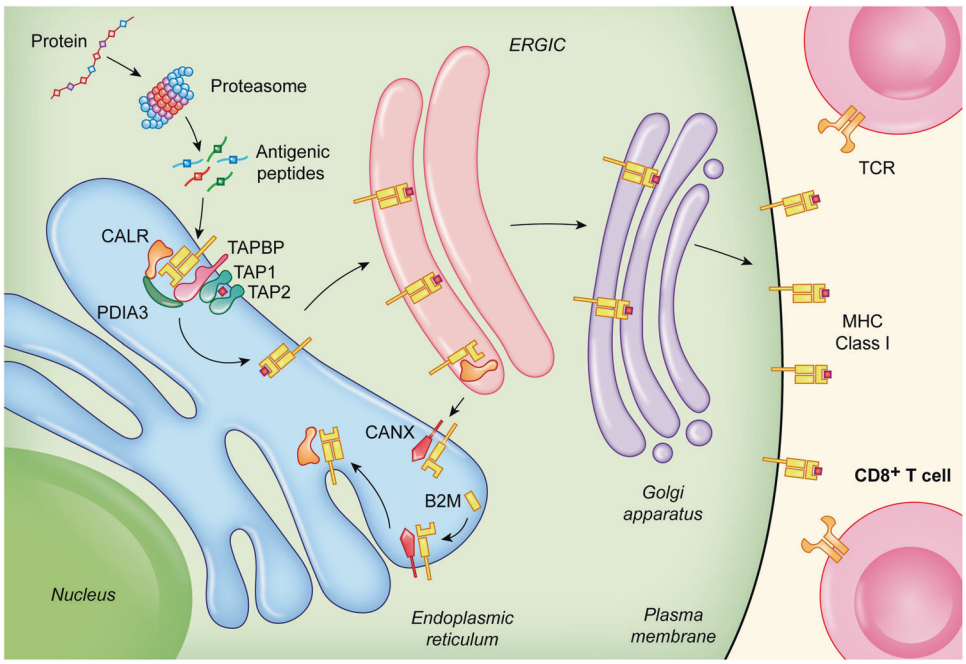

Fig. 2 Calreticulin in antigen presentation. CALR, CANX and PDIA3 are key to the proper assembly of MHC Class I heavy chains with B2M in the ER lumen, as well as to (1) the loading of antigenic peptides onto mature MHC Class I molecules and (2) the retrieval of sub-optimally assembled MHC Class I molecules from post-ER compartments, notably the ERGIC and the *cis*-Golgi. TCR, T-cell receptor.

(IFNγ) produced by type I helper (T_H1) $CD4^+$ cells to overcome the defect in MHC Class I presentation associated with *CALR* mutations.[71]

Taken together, these findings suggest that shared mutations in *CALR* exon 9 are amenable to targeting by immunotherapy, and a phase I clinical trial testing a therapeutic peptide-based vaccine specific for mutant CALR in subjects with MPN is ongoing (NCT03566446).[72] Since *CALR* mutations are the second most common MPN drivers and the TNAs they generate are shared among different patients, this therapeutic strategy, if successful, may be beneficial for a significant number of patients with MPN.[35,73]

CALR IN DANGER SIGNALING

Preclinical and clinical data accumulating over the past decade indicate that besides playing a key role in the maintenance of reticular homeostasis and in antigen presentation, CALR is a major determinant of cellular adjuvanticity, i.e., the ability of stressed and dying cells to deliver co-stimulatory (rather than co-inhibitory) signals to immune cells[11,12] (Fig. 3). This function does not originate from physiological CALR pools within the ER or ERGIC, but from an expanded pool of CALR molecules that are exposed on the membrane of cells undergoing the so-called integrated stress response (ISR),[74] a multipronged, cell-wide reaction to specific perturbations of the extracellular or intracellular microenvironment that cause (in a majority of cases) the loss of ER homeostasis.[75,76] In particular, the translocation of CALR on the outer surface of the plasma membrane, which relies on the inactivating phosphorylation of eukaryotic translation initiation factor 2 subunit alpha (EIF2S1, best known as eIF2α)[77] (Box 2), can be induced by a number of chemotherapeutic agents (i.e., anthracyclines, taxanes, oxaliplatin, bortezomib, PT-112, and others),[78–84] radiation therapy,[85,86] some variants of photodynamic therapy,[87–91] high hydrostatic pressure,[92,93] oncolytic peptides,[94–96] and oncolytic viruses,[97–101] among others.[102,103]

Cell surface-exposed CALR delivers potent pro-phagocytic signals to APCs including DCs and their precursors,[104,105] de facto initiating the uptake of dying cells or their corpses in the context of immunostimulation.[106,107] This process is a conditio sine qua non for the initiation of a tumor-targeting immune response by cancer cells undergoing ICD.[11,12] In line with this notion, wild-type mouse cancer cells exposed to an ICD inducer in vitro can be used as vaccines to establish long-term prophylactic immunity against living cancer cells of the same type (in immunocompetent syngeneic mice), but lose their vaccination potential when CALR is lost, downregulated or blocked.[74,89,108] Supporting a specific role for surface-exposed (as opposed to reticular) CALR in this setting, surface adsorption of recombinant CALR generally restores the prophylactic power of CALR-depleted cancer cells undergoing ICD in vitro.[74,108] Importantly, CALR exposure on the surface of cells undergoing ICD is generally required, but not sufficient, for cell death to be perceived as immunogenic, as dying cells must also express antigens that are not covered by central or peripheral tolerance in a specific host and emit several other adjuvant-like signals, collectively known as damage-associated molecular patterns (DAMPs),[109] as they die.[11] These signals, which are decoded by pattern recognition receptors expressed by immune cells,[110] include, but are not limited to: (1) ATP secretion, favoring the recruitment of DCs and their precursors to sites of ICD, and their activation;[111] (2) the release of the nuclear protein high mobility group Box 1 (HMGB1), which mediates immunostimulatory functions;[112] (3) the secretion of annexin A1 (ANXA1), which directs the interaction of DCs to dying cells or their corpses;[113] and (4) the synthesis and secretion of type I interferon (IFN), which amplifies local immunostimulation via cancer cell-intrinsic and -extrinsic mechanisms.[114–117] Finally, the site of cell death must be compatible with the initiation of an immune response, and hence be scarcely infiltrated by immunosuppressive cells but accessible to myeloid and lymphoid immune effectors.[11]

The pro-phagocytic effects of surface-exposed CALR have largely been attributed to LDL receptor-related protein 1 (LRP1, best known as CD91) on the surface of APCs.[118] However, additional proteins that bind extracellular CALR have been described, including thrombospondin 1 (THBS1), which has been proposed to cooperate with CALR in the regulation of integrin-

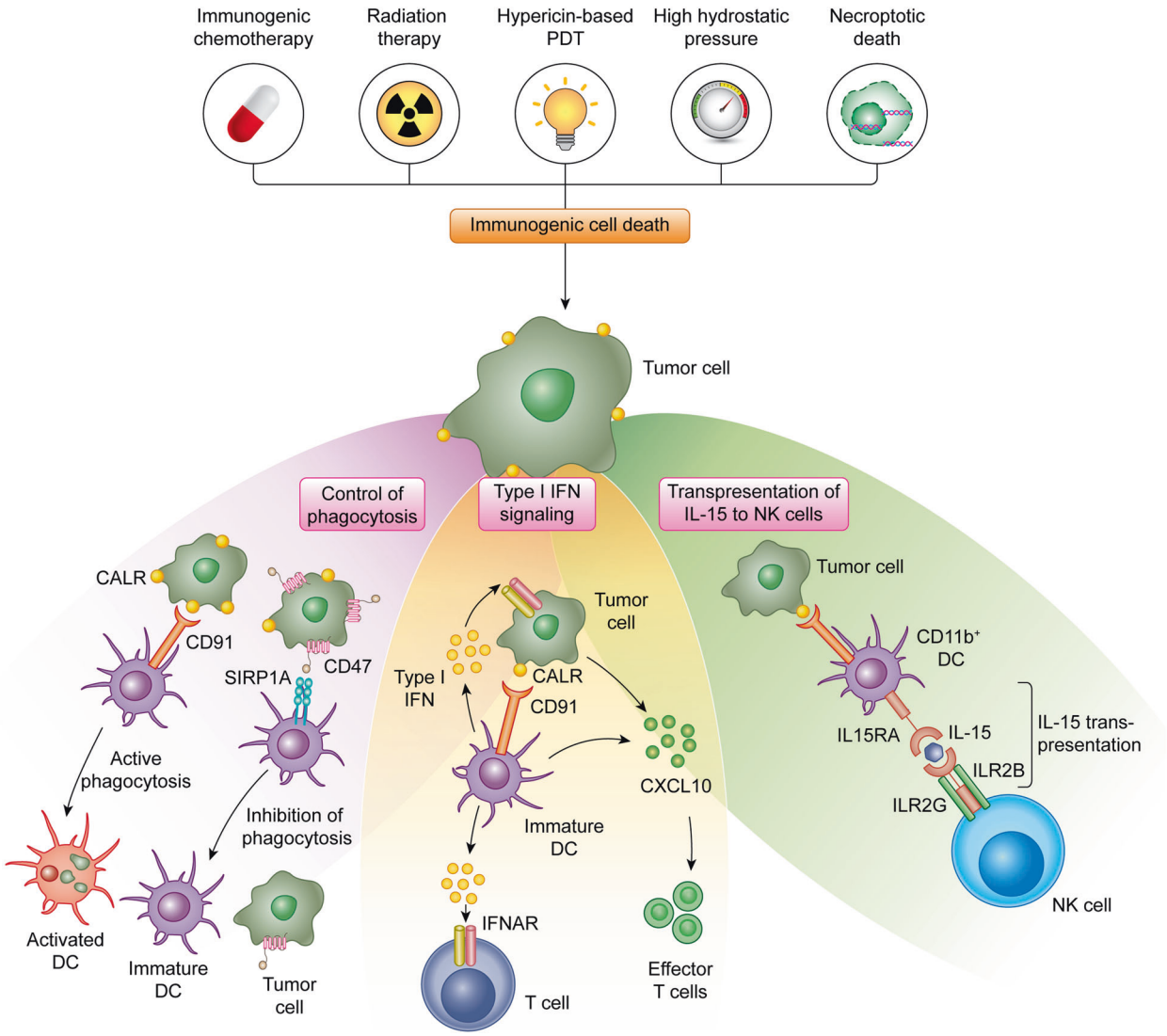

Fig. 3 Calreticulin in danger signaling. The exposure of CALR on the surface of stressed and dying tumor cells mediate multipronged immunostimulatory effect. First, surface-exposed CALR promotes the uptake of dying cells or their corpses by DCs, a process that is actively inhibited by CD47. Second, the exposure of CALR has been associated with the activation of an IFN response in dying cells, although the underlying mechanisms remain to be elucidated. Third, surface-exposed CALR promotes the expansion of $CD11b^{+}CD14^{+}$ monocytes that proficiently *trans*-present IL15 to NK cells. CXCL10, C-X-C motif chemokine ligand 10; IL2RB, interleukin 2 receptor subunit beta; IL2RG, interleukin 2 receptor subunit gamma; PDT, photodynamic therapy; SIRP1A, signal regulatory protein alpha.

independent cell adhesion;[119,120] complement C1q A chain (C1QA), which appears to harness CALR as a receptor for cell surface binding;[121] as well as members of the mannose-binding lectin (MBL) family.[122] Of note, both C1QA and lectin, mannose binding 1 (LMAN1, also known as MBL1) have also been proposed to support the CALR-dependent phagocytosis of apoptotic bodies.[123,124] The potential involvement of CD91 in these latter processes, however, remains to be clarified.

The pro-phagocytic effects of surface-exposed CALR are counteracted by at least two different mechanisms. On the one hand, cells undergoing caspase-dependent apoptosis generally expose abundant amounts of phosphatidylserine (PS), a phospholipid normally confined to the inner leaflet of the plasma membrane, on their surface.[125–127] Surface-exposed PS can be rapidly recognized by the receptor jumonji domain-containing 6, arginine demethylase and lysine hydroxylase (JMJD6, best known as PSR), which initiates the rapid, immunologically silent clearance of cell corpses by macrophages.[128–130] Thus, CALR must be exposed prior to PS for cell death to be perceived as immunogenic.[77,131] On the other hand, the phagocytosis of dying cells is actively inhibited by CD47, an integrin-associated protein that operates by binding signal regulatory protein alpha (SIRPA) on the surface of APCs.[132–134] Thus, the relative levels of surface-exposed CALR and CD47 dictate the ultimate outcome of the physical interaction between dying cells and phagocytes.[135] Multiple companies are developing CD47- or SIRPA-targeting antibodies to promote the phagocytosis of dying cancer cells in the context of immunostimulatory signals that support therapeutically relevant tumor-targeting immunity.[136] The clinical potential of these agents, however, remains to be clarified.

Interestingly, surface-exposed CALR seems to mediate immunostimulatory effects that are not directly related to the phagocytosis of dying cells. In particular, high levels of CALR on the surface of AML blasts, which correlates with signs of an ongoing ISR, favors the accumulation of a population of $CD11b^{+}CD14^{+}$ myeloid cells that express high levels of maturation markers and interleukin 15 receptor, alpha chain (IL15RA).[137] This endows $CD11b^{+}CD14^{+}$ myeloid cells with the ability to

Box 2 Molecular mechanisms of CALR exposure

CALR is translocated from the ER lumen to the outer leaflet of the plasma membrane in cells responding to specific microenvironmental perturbations (either productively, i.e., as they survive to stress, or non-productively, i.e., as they succumb to stress) via a tri-modular mechanism inserted into the so-called integrated stress response (ISR).[75] The first functional module is strictly related to the ISR and involves the inactivating phosphorylation of eukaryotic translation initiation factor 2 subunit alpha (EIF2S1, best known as eIF2α) by eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3, best known as PERK).[77] Consistent with this, CALR exposure driven by anthracyclines is abolished in mouse colorectal carcinoma CT26 cells depleted of PERK by RNA interference or expressing a non-phosphorylatable variant of eIF2α.[77] The second functional module is related to apoptotic signaling,[202] as it involves the pro-apoptotic Bcl-2 family members BCL2 associated X, apoptosis regulator (BAX) and BCL2 antagonist/killer 1 (BAK1), caspase 8 (CASP8) as well as the CASP8 substrate B-cell receptor-associated protein 31 (BCAP31, also known as BAP31).[77] Thus, anthracycline-driven CALR exposure is lost in CT26 cells depleted of BAX, BAK1 or CASP8, as well as in CT26 cells expressing a non-cleavable variant of BAP31[77] or exposed to a pan-caspase inhibitor.[78] The third module involves the machinery for anterograde ER-to-Golgi transport.[203,204] In line with this notion, knockdown of vesicle-associated membrane protein 1 (VAMP1) or synaptosome-associated protein 25 (SNAP25) in CT26 cells inhibits CALR exposure driven by anthracyclines. Of note, at least in some cancer cells including CT26 cells, CALR translocation to the outer leaflet of the plasma membrane obligatorily relies on the co-translocation of protein disulfide isomerase family A member 3 (PDIA3).[131] That said, it is probable that the molecular machinery for CALR exposure exhibits at least some degree of heterogeneity linked to cell type and initiating trigger.

trans-present interleukin 15 (IL15) to natural killer (NK) cells, de facto boosting their effector functions against AML cells.[137,138] Moreover, CALR exposure on the surface of AML cells has been associated with signatures of type I IFN signaling,[137,139] and the immunological control of AML cells engineered to constitutively translocate CALR to the outer leaflet of the plasma membrane is abrogated in mice lacking interferon alpha and beta receptor subunit 1 (IFNAR1).[139] Although the molecular mechanisms linking CALR exposure and type I IFN signaling in AML blasts have not yet been elucidated, these findings point to a functional link between two different ICD-relevant DAMPs.

Importantly, both type I and type II mutations in exon 9 compromise the KDEL domain of CALR, which normally enables its traffic from the ER to the Golgi apparatus and back with limited surface exposure or extracellular secretion.[48] In line with this notion, cancer cells bearing type I *CALR* mutations (i.e., del52) secrete increased amount of CALR as compared to their wild-type counterparts.[140] Moreover, patients with MPNs driven by *CALR* exon 9 mutations display elevated plasma levels of CALR as compared to healthy individuals.[140] Mechanistically, soluble CALR acts as a decoy for CALR receptors in APCs, de facto limiting the uptake of dying cancer cells and their ability to initiate protective immunity in immunocompetent hosts, correlating with the accumulation of immunosuppressive cells in the spleen and peripheral blood.[140] CALR secretion as a consequence of KDEL loss also compromises the therapeutic response of AML to immune checkpoint blockers targeting PD-1, at least in mice.[140] In summary, while wild-type CALR operates as a key DAMP in the context of ICD, its mutant counterpart prevents the initiation of tumor-targeting immunity, which identifies yet another immunological advantage conveyed by *CALR* mutations to cancer cells.

PROGNOSTIC AND PREDICTIVE VALUE OF CALR

Accumulating data lend further support to the notion that wild-type CALR mediates oncosuppressive effects by limiting malignant features and/or enabling the immunosurveillance of developing tumors (Table 1).[17]

Low CALR levels have been associated with accrued malignant features including hyperproliferation in preclinical models of prostate cancers[141] as well as advanced disease stage in 128 patients with urothelial carcinoma.[65] Along similar lines, robust CALR expression in diagnostic biopsies have been linked with improved disease outcome in 68 patients with colorectal carcinoma,[142] 68 subjects with neuroblastoma,[143] three independent cohorts of 270, 125 and 23 individuals with non-small cell lung carcinoma (NSCLC),[108,144] 105 patients with AML,[145] 30 subjects with osteosarcoma,[146] 9 patients with glioblastoma,[147] as well as in three independent cohorts of 152, 202 and 302 women with ovarian carcinoma.[108,148] In many of these settings, high CALR levels correlated with activation of the ISR,[145,148] and/or one or multiple signs of ongoing anticancer immunity, including infiltration by $CD45RO^+$ memory T cells in colorectal carcinoma,[142] and abundant intratumoral levels of DCs and T_H1 $CD4^+$ T cells in NSCLC.[144] Similarly, abundant CALR exposure on the surface of malignant cells has been linked to superior disease outcome in two independent cohort of 50 and 20 patients with AML, correlating with increased levels of T cells specific for tumor-associated antigens[149] or limited CD47 expression.[150] Importantly, these latter studies were performed in chemotherapy-naïve patients,[149,150] suggesting that AML blasts can spontaneously expose CALR on their surface (at least in some settings), potentially as a cellular response to oncogenic stress or adverse microenvironmental conditions in the leukemic marrow.[151]

Apparently at odds with the above, high CALR levels in diagnostic biopsies have also been associated with negative prognostic value in some patient cohorts (Table 1). In particular, robust CALR expression has been correlated with rapid tumor progression and poor disease outcome in 79 patients with gastric carcinoma,[152] 58 subjects with NSCLC,[153] two independent cohorts of 228 and 33 women with breast carcinoma,[154,155] two independent sets of 478 and 251 individuals with neuroblastoma,[135] two independent cohorts of 80 and 68 patients with pancreatic cancer,[156,157] two independent sets of 165 and 30 subjects with bladder carcinoma,[135] and two independent cohorts of 92 and 71 patients with mantle cell lymphoma.[135] These findings suggest that the intracellular functions of CALR as a key regulator of Ca^{2+} homeostasis and integrin-dependent signaling may be required for some tumors to progress. Alternatively, the negative prognostic impact of robust CALR expression in some oncological settings may originate from the compensatory overexpression of CD47, as documented in AML, acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL) samples.[135] Supporting this possibility, elevated CD47 levels have been linked to poor disease outcome in 132 patients with karyotypically normal AML,[158] 265 women with ovarian carcinoma,[159] and 102 individuals with esophageal squamous cell carcinoma.[160]

Recently, somatic frameshift mutations in *CALR* exon 9 have been identified in a large proportion of patients with primary myelofibrosis (PMF), essential thrombocythemia (ET) or polycythemia vera (PV) bearing wild-type *JAK2* and *MPL*.[35] These patients are younger and exhibit lower hemoglobin levels, decreased leukocytosis and higher platelet counts as compared to their *JAK2*- and *MPL*-mutant counterparts.[161] Similarly, PMF and ET patients bearing *CALR* mutations are younger, and display lower incidence of anemia and leukocytosis, lower Dynamic International Prognostic Scoring System (DIPSS) score and reduced frequency of spliceosome mutations, as well as higher platelet counts than patients with wild-type JAK2, MPL and CALR, de facto experiencing improved disease outcome (median survival: 16 vs 2.3 years).[162,163] Altogether, these findings document that CALR mutations constitute a positive prognostic factor in patients with MPN and a valuable predictive biomarker for allocating these patients to allogenic hematopoietic stem cell transplantation.[164] Consistent with this, *CALR* mutational status has recently been incorporated into numerous PMF scoring systems, including the MYelofibrosis Secondary to PC and ET Prognostic Model (MYSEC-PM), the Mutation-enhanced International Prognostic Scoring System for transplant-eligible patients (MIPSS70), and the

Table 1. Prognostic impact of CALR in cancer patients.

Cancer type	No. of patients	Therapy	Detection	Impact	Ref.
AML	20	Anthracyclines	FC	↑ CALR exposure on blasts correlated with improved RFS	150
AML	50	Anthracyclines	FC	↑ CALR exposure on blasts correlated with improved RFS and OS	149
AML	105	Cytarabine and anthraclycines	RT-PCR	↑ CALR levels correlated with improved, DFS, RFS and OS	145
Bladder	30	Chemotherapy	Microarray	↑ CALR levels correlated with poor DSS	135
Bladder	165	Surgery, chemotherapy	Microarray	↑ CALR levels correlated with poor OS	135
Breast	23	Surgery	IHC	↑ CALR levels correlated with poor MFS	155
Breast	228	n.a.	IHC	↑ CALR levels associated with advanced stage and metastatic dissemination	154
Colorectal	68	Surgery, chemotherapy	IHC	↑ CALR levels correlated with improved OS	142
ET	292	n.a	PCR	*CALR* mutations correlated with improved OS	163
ET	576	n.a.	PCR	*CALR* mutations correlated with limited incidence of thrombosis	162
ET	745	n.a.	PCR	*CALR* mutations correlated with limited incidence of thrombosis	161
Gastric	79	Surgery	IHC	↑ CALR levels correlated with poor disease outcome	152
Glioblastoma	9	Radiation therapy and temozolomide	IHC	↑ CALR levels correlated with improved OS	147
MCL	71	None	Microarray	↑ CALR levels correlated with poor OS	135
MCL	92	Chemotherapy	Microarray	↑ CALR levels correlated with poor OS	135
Neuroblastoma	68	Surgery, chemotherapy	IHC	↑ CALR levels correlated with improved disease outcome	143
Neuroblastoma	251	Surgery, chemotherapy	Microarray	↑ CALR levels correlated with poor OS	135
Neuroblastoma	478	Surgery, chemotherapy	Microarray	↑ CALR levels correlated with poor OS	135
NSCLC	23	Radiation therapy	Microarray	↑ CALR levels correlated with improved OS	108
NSCLC	58	Chemotherapy	IHC	↑ CALR levels correlated with advanced stage	153
NSCLC	125	Surgery, chemotherapy	IHC	↑ CALR levels correlated with improved OS	144
NSCLC	270	Surgery	IHC	↑ CALR levels correlated with improved OS	144
Osteosarcoma	30	Chemotherapy	IF	↑ CALR levels correlated with local (vs metastatic) disease	146
Ovarian	152	Surgery, chemotherapy	IHC	↑ CALR levels correlated with improved RFS and OS	148
Ovarian	220	Chemotherapy	Microarray	↑ CALR levels correlated with improved DFS and OS	108
Ovarian	302	Surgery, chemotherapy	Microarray	↑ CALR levels correlated with improved OS	148
Pancreatic	68	Surgery	IHC	↑ CALR levels correlated with poor disease outcome	157
Pancreatic	80	Surgery	IHC	↑ CALR levels correlated with poor disease outcome	156
PMF	133	Allogeneic HSCT	PCR	*CALR* mutations correlated with improved OS	164
PMF	267	n.a	PCR	*CALR* mutations correlated with improved OS	163
PV	490	n.a.	PCR	*CALR* mutations correlated with limited incidence of thrombosis	161
Urothelial	128	Surgery, chemotherapy, radiation therapy	IHC	↑ CALR levels correlated with improved OS	65

AML acute myeloid leukemia, *CALR* calreticulin, *DFS* disease-free survival, *DSS* disease-specific survival, *ET* essential thrombocythemia, *FC* flow cytometry, *HSCT* hematopoietic stem cell transplantation, *IHC* immunohistochemistry, *MCL* mantle cell lymphoma, *MFS* metastasis-free survival, *n.a.* not available, *NSCLC* non-small cell lung carcinoma, *OS* overall survival, *PMF* primary myelofibrosis, *PV* polycythemia vera, *RFS* relapse-free survival.

Genetically Inspired Prognostic Scoring System (GIPSS).[165–167] Conversely, the *CALR*, *JAK2* and *MPL* mutational status does not appear to influence the survival of patients with ET.[161] The immunobiological bases for this discrepancy remain to be elucidated.

CALR mutations yielding a secreted protein that serves as a decoy to saturate CALR receptors, hence subverting the immune recognition of CALR-exposing stressed and dying tumor cells, have also been documented in ~1%–2% of solid neoplasms.[140] Although this percentage is insufficient for prognostic or predictive assessments, it is tempting to speculate that such mutations, which preferentially occur in the C-terminus of the protein,[140] may subvert immunosurveillance and favor tumor progression.

In summary, CALR appears to influence malignant transformation, disease progression and response to therapy in various tumor types, standing out as a prominent prognostic factor in multiple oncological settings. To harness the prognostic and potentially predictive potential of CALR, however, it will be important to identify and exclude potential confounders, such as CD47 expression.

CONCLUDING REMARKS

CALR influences a variety of processes that are key to organismal homeostasis, including (but presumably not limited to) protein folding, Ca^{2+} homeostasis, cellular adhesion, motility, antigen presentation and danger signaling. It is therefore not surprising

that loss-of-function *CALR* mutations favor (at least in some cases) oncogenesis, tumor progression and resistance to treatment.

At least in part, this scenario is reminiscent of tumor protein p53 (*TP53*).[168] TP53 (best known as p53) mediates indeed a variety of oncosuppressive functions that span from the regulation of metabolism, redox homeostasis and cell fate in (pre)neoplastic cells to the initiation of innate and adaptive anticancer immune responses.[168] However, p53 is often inactivated as a consequence of point mutations or hyperactivation of the p53-degrading enzyme MDM2 proto-oncogene (MDM2), which (at least theoretically) can be targeted pharmacologically.[169,170] Conversely, *CALR* mutations are small indels that compromise the C-terminus of the protein, and hence appear difficult to target with pharmacological interventions. Potentially, patients with MPNs bearing *CALR* mutations may benefit from agents that specifically disrupt CALR–MPL interactions.[171] However, the development of such molecules is still in its infancy.

Finally, while the cancer cell-intrinsic functions of CALR may support tumor progression at least in some settings, pharmacological inhibitors affecting the reticular functions of CALR employed as systemic agents may have considerable side effects, in line with the key role of the wild-type protein in several adult tissues (see above). This situation is reminiscent of autophagy. Autophagy is an evolutionarily conserved mechanism for the preservation of cellular and organismal homeostasis that some tumors harness in support of disease progression and resistance to treatment.[76] However, autophagy is key to the physiological functions of many tissues, notably the brain, which complicates considerably the use of autophagy inhibitors delivered systemically for cancer therapy.[172]

Thus, for the time being, CALR stands out mostly as a promising prognostic and/or predictive factor (rather than as a therapeutic target), especially for patients with MPNs. As a potential exception, a phase I clinical trial is currently investigating the safety and preliminary efficacy of a therapeutic peptide-based vaccine specific for mutant CALR in subjects with MPN (NCT03566446). Until now, however, therapeutic peptide-based vaccination has demonstrated limited efficacy in clinical settings,[173] which dampens (at least to some degree) enthusiasm on the possibility to treat MPN with a peptide-based vaccine specific for mutant CALR. Additional work is urgently required to devise a therapeutic strategy to target CALR in cancer.

ACKNOWLEDGEMENTS

G.K. is supported by the Ligue contre le Cancer (équipe labellisée); Agence National de la Recherche (ANR) — Projets blancs; ANR under the frame of E-Rare-2, the ERA-Net for Research on Rare Diseases; Association pour la recherche sur le cancer (ARC); Cancéropôle Ile-de-France; Chancelerie des universités de Paris (Legs Poix), Fondation pour la Recherche Médicale (FRM); a donation by Elior; European Research Area Network on Cardiovascular Diseases (ERA-CVD, MINOTAUR); Gustave Roussy Odyssea, the European Union Horizon 2020 Project Oncobiome; Fondation Carrefour; High-end Foreign Expert Program in China (GDW20171100085 and GDW20181100051), Institut National du Cancer (INCa); Inserm (HTE); Institut Universitaire de France; LeDucq Foundation; the LabEx Immuno-Oncology; the RHU Torino Lumière; the Seerave Foundation; the SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); and the SIRIC Cancer Research and Personalized Medicine (CARPEM). L.G. is supported by a Breakthrough Level 2 grant from the US Department of Defense (DoD), Breast Cancer Research Program (BRCP) (#BC180476P1), by the 2019 Laura Ziskin Prize in Translational Research (#ZP-6177, PI: Formenti) from the Stand Up to Cancer (SU2C), by a Mantle Cell Lymphoma Research Initiative (MCL-RI, PI: Chen-Kiang) grant from the Leukemia and Lymphoma Society (LLS), by a startup grant from the Department of Radiation Oncology at Weill Cornell Medicine (New York, USA), by a Rapid Response Grant from the Functional Genomics Initiative (New York, USA), by industrial collaborations with Lytix (Oslo, Norway) and Phosplatin (New York, USA), and by donations from Phosplatin (New York, US), the Luke Heller TECPR2 Foundation (Boston, USA) and Sotio a.s. (Prague, Czech Republic).

AUTHOR CONTRIBUTIONS

J.F. and L.G. conceived the manuscript. J.F. and L.G. wrote the first version of the manuscript with constructive input from R.S. and G.K. J.F. prepared display items under supervision from L.G. L.G. addressed issues raised by reviewers and requested by editors. All authors approved the final version of the article.

ADDITIONAL INFORMATION

Competing interests: J.F. and R.S. are full-time employees of Sotio. G.K. has been holding research contracts with Bayer Healthcare, Genentech, Glaxo Smyth Kline, Institut Mérieux, Lytix Pharma, PharmaMar, Sotio and Vasculox. He is on the Board of Directors of the Bristol Myers Squibb Foundation France, member of the Scientific Advisory Board of The Longevity Labs, and scientific co-founder of everImmune, Samsara therapeutics and Therafast Bio. L.G. received research support from Lytix and Phosplatin, consulting fees from OmniSEQ, Astra Zeneca, Inzen and the Luke Heller TECPR2 Foundation, and he is member of Scientific Advisory Committees for Boehringer Ingelheim, The Longevity Labs and OmniSEQ.

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