Apoptotic caspases inhibit abscopal responses to radiation and identify a new prognostic biomarker for breast cancer patients

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ABSTRACT
Caspase 3 (CASP3) has a key role in the execution of apoptosis, and many cancer cells are believed to disable CASP3 as a mechanism of resistance to cytotoxic therapeutics. Alongside, CASP3 regulates stress-responsive immunomodulatory pathways, including secretion of type I interferon (IFN). Here, we report that mouse mammary carcinoma TSA cells lacking Casp3 or subjected to chemical caspase inhibition were as sensitive to the cytostatic and cytotoxic effects of radiation therapy (RT) in vitro as their control counterparts, yet secreted increased levels of type I IFN. This effect originated from the accrued accumulation of irradiated cells with cytosolic DNA, likely reflecting the delayed breakdown of cells experiencing mitochondrial permeabilization in the absence of CASP3. Casp3-/- TSA cells growing in immunocompetent syngeneic mice were more sensitive to RT than their CASP3-proficient counterparts, and superior at generating bona fide abscopal responses in the presence of an immune checkpoint blocker. Finally, multiple genetic signatures of apoptotic proficiency were unexpectedly found to have robust negative (rather than positive) prognostic significance in a public cohort of breast cancer patients. However, these latter findings were not consistent with genetic signatures of defective type I IFN signaling, which were rather associated with improved prognosis. Differential gene expression analysis on patient subgroups with divergent prognosis (as stratified by independent signatures of apoptotic proficiency) identified SLC7A2 as a new biomarker with independent prognostic value in breast cancer patients. With the caveats associated with the retrospective investigation of heterogeneous, public databases, our data suggest that apoptotic caspases may influence the survival of breast cancer patients (or at least some subsets thereof) via mechanisms not necessarily related to type I IFN signaling as they identify a novel independent prognostic biomarker that awaits prospective validation.

Introduction
Apoptosis is a variant of regulated cell death (RCD) that relies on the sequential activation of two or more cysteine-dependent aspartate-directed proteases commonly known as caspases. Apoptosis not only provides a major contribution to the fully physiological waves of cellular demise that underlie both (post-)embryonic development and adult tissue homeostasis, but is also responsible for the death of malignant cells exposed to adverse microenvironmental conditions or exposed to cytotoxic therapeutic regimens, including chemotherapy and radiation therapy (RT). In line with this notion, the ability of progressing tumors to acquire genetic or epigenetic alterations to circumvent cell survival (or not) by cancer cells, be they successful (i.e., culminating with cell death), and the configuration of the tumor microenvironment. In this setting, apoptotic caspases – namely, CASP3, CASP6, CASP7, CASP8, CASP9 and CASP10 – turned out to occupy a key position in the control of signals whereby stress (cancer) cells communicate with the immunological microenvironment.

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CASP3 (the main executor of both intrinsic and extrinsic variants of apoptosis) stands out as a major contributor to the establishment of peripheral tolerance against dying cells, at least in part reflecting the need for suppressed immunity in the context of physiological cell death.\textsuperscript{2,3,12} Indeed, CASP3 activation drives the exposure of phosphatidylserine (PS) on the outer leaflet of dying cells, which favors the phagocytic activity of macrophages in the context of immunosuppressive signaling.\textsuperscript{13-16} It stimulates the secretion of prostaglandin E2 (PGE\textsubscript{2}), which actively inhibits immune effector cells,\textsuperscript{17,18} and that of lysophosphatidylcholine (LPC), which recruits phagocytes to sites of cell death to favor removal of cell corpses in the absence of inflammation.\textsuperscript{19} Moreover, activation of CASP3 downstream of mitochondrial outer membrane permeabilization (MOMP) and consequent CASP9 activation inhibits the secretion of type I interferon (IFN) driven by the ability of cyclic GMP-AMP synthase (CGAS) to detect mitochondrial DNA (mtDNA) accumulating in the cytoplasm.\textsuperscript{20-23}

CGAS signaling and consequent secretion of type I IFN by irradiated cells are paramount for abscopal responses (i.e., the immunological control of out-of-field malignant lesions following the irradiation of an anatomically distant tumor).\textsuperscript{24} We therefore set to elucidate the impact of apoptotic caspase activation on type I IFN secretion and responses to RT in a transplantable model of mammary breast carcinoma (TSA cells). Our preclinical findings demonstrate that CASP3 inhibits abscopal responses by accelerating the breakdown of cells that accumulate cytosolic DNA in response to RT, pointing to CASP3 as an actionable target to ameliorate the clinical efficacy of RT. However, bioinformatic analyses on publicly available datasets suggest that type I IFN signaling has a negative impact on the survival of breast cancer patients. Moreover, differential gene expression studies on patient subgroups exhibiting divergent prognosis (as stratified by independent signatures of apoptotic proficiency) identified 11 common genes, 3 of which had never been attributed an impact on survival in this setting. Of these 3 genes, solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 (SLC7A2) had independent prognostic value on multivariate Cox regression analyses. With the caveats linked to retrospective bioinformatic studies of heterogeneous, publicly available databases, our data suggest that apoptotic caspases may influence the survival of breast cancer patients (or at least some subsets thereof) via mechanisms not necessarily related to type I IFN signaling, as they identify a novel, independent prognostic biomarker that awaits prospective validation.

### Materials and methods

#### Chemicals and cell culture

Unless otherwise specified, chemicals have been acquired from Sigma-Aldrich, reagents for cell culture from Gibco®-Thermo Fischer, and plasticware from Corning. Wild-type mouse breast carcinoma TSA cells, as well as control and Casp3\textsuperscript{-/-} TSA cell clones were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), as per ATCC recommendations.

Cells between passage 2 and 10 were employed for experimental determinations. Control and Casp3\textsuperscript{-/-} TSA clones were generated via the CRISPR/Cas9 technology based on commercial non-targeting and CASP3-specific guide RNAs from Sigma-Aldrich. Plasmid transfection and clone selection were performed as per the manufacturer’s recommendations.

#### Flow cytometry

Flow cytometry was used to determine MOMP, PS exposure and plasma membrane rupture as per standard protocols.\textsuperscript{25} In brief, MOMP was monitored with the mitochondrial transmembrane potential-sensitive dye 3,3′-dihexyloxacarbocyanine iodide (DiOC\textsubscript{6}(3), from Invitrogen®-Thermo Fischer) (40 nM), PS exposure with FITC-conjugated annexin V (Biolegend), and plasma membrane rupture with propidium iodide (PI, from Sigma-Aldrich) (0.5 µg/mL). Stained samples were acquired on a MACSQuant® Analyzer 10 (Miltenyi Biotech) and data were analyzed with FlowJo v. 10.6 (FlowJo LLC).

#### Immunoblotting

Immunoblotting was performed as previously reported,\textsuperscript{26} with primary antibodies specific for CASP3 (#14220 from Cell Signaling Technology), MAPLC3B (#2775, from Cell Signaling Technology), SQSTM1 (#5114S, from Cell Signaling Technology), or actin, beta (ACTB, #8H10D10, from Cell Signaling Technology), which was employed as loading control. Upon wash and incubation with species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (from GE Healthcare Life Sciences), revelation was performed with ECL Select Detection Reagent (from GE Healthcare Life Sciences) on a C6000 Gel Doc & Western Imaging System (Azure Biosystems).

#### Immunofluorescence

For immunofluorescence, cells growing on glass coverslip were fixed and permeabilized with 0.1% Tween20 and 0.01% Triton X-100 in PBS, followed by incubation with primary antibodies specific for double-stranded DNA (dsDNA, #ab27156, from Abcam), wash and incubation with species-specific secondary antibodies conjugated to Alexa Fluor® 594 (ab150120, from Abcam).\textsuperscript{27} Samples were mounted on slides with 4',6-diamidino-2-phenylindole (DAPI)-containing ProLong™ Diamond Antifade Mountant (from Thermofischer), and images were acquired with an Evos® FL Imaging System (Thermo Fischer). dsDNA quantification was performed on 8–10 randomly selected fields per sample, with ImageJ (NIH).

#### RT-PCR

IFNB1 levels were quantified relative to RPL19 levels, according to standard RT-PCR procedures based on commercial primer sets from Bio-Rad. Amplifications were run on a 7500 RT-PCR system (Applied Biosystems) as per thermal protocols provided by the primer manufacturer, and data were analyzed with the ΔΔC\textsubscript{t} method.
**ELISA**

Type I IFN levels in the culture supernatants were quantified with the VERIKINE-HS™ IFN Beta Serum ELISA kit (#42410, from PBL Assay Science), as per the recommendations of the manufacturer.

**In vivo studies**

Female 4–9-weeks old BALB/c were employed as host for single neoplastic lesions driven by $0.25 \times 10^6$ wild-type TSA cells implanted s.c. in one flank, or double neoplastic lesions driven by $0.25 \times 10^6$ control TSA cells implanted s.c. contralaterally. Mice were monitored for tumor growth on the primary (in both experimental settings) and abscopal site (in the double lesion model only) routinely by means of common caliper, and once primary tumors reached a surface area of $15–25$ mm$^2$ (day 0), mice were allocated to either of the following treatment groups: (1) control, no treatment or 100 µL vehicle i.p. on days 2, 5 and 8; (2) a single RT fraction of 20 Gy on day 0; (3) 3 RT fractions of 8 Gy each on days 0, 1 and 2; and (4) 3 RT fractions of 8 Gy each on days 0, 1 and 2, combined with a cytotoxic T lymphocyte associated protein 4 (CTLA4)-targeting antibody ($9H10$, from Bio X Cell), 200 µg/mouse in 100 µL i.p., on days 2, 5 and 8. RT was delivered with the Small Animal Radiation Research Platform (SARRP, from Xstrahl). Response to treatment and potential toxicities were monitored routinely. Mice experiencing complete tumor regression were re-challenged with wild-type TSA cells.

**Bioinformatic analyses**

All statistical analyses and graphics were performed in the statistical programming language R (version 3.5.2). Gene expression levels and clinicopathological parameters of 1422 breast cancer patients (METABRIC database) for which cancer-specific overall survival was available were acquired from cBioportal (http://www.cbioportal.org/). Survival analysis was performed using the Survival and Survminer R packages. The Kaplan–Meier method was used to estimate patient overall survival rates upon patient stratification into 2 groups based on median mRNA levels of individual genes (AGR3, ANKRD30A, APAF1, BMRR1B, CALML5, CASP3, CASP9, CLIC6, CYP4X1, CXX9, LTB, SLC7A2, STC2, SUSD3) or the following signatures, calculated as geometric mean of respective genes: BCLs (BCL2, BCL2L1, MCL1), type I IFN signaling (ADAR, B2M, BAX, B2M, CASP1, CASP8, CCL2, C4D, CD74, CMPK2, CNP, CSF1, CXCL10, CXCL11, DDX60, DHH5X8, EIF2AK2, EIF1, EPSTI1, FM125A, FAM46A, FTSJ1D2, GBP2, GBP4, GMPR, HERC6, HLA-C, IFI27, IFI30, IFI35, IFI44, IFI44L1, IFI41, IFI42, IFI43, IFITM1, IFITM2, IFITM3, IL15, IL18, IRF1, IRF2, IRF7, IRF9, ISG15, ISG20, LAG3, LAMC3, LCP3, LGALS3BP, LYSMD2, MARCH1, METTL7B, MTA2, MTHFD2, MVX, MX1, MX2, MYD88, NAMPT, NCOA3, NFKB1, NFKB1A, NR5C, NMI, NOD1, NUP93, OAS2, OAS3, OASL, OGER, P2Y14, PARP12, PARP14, PDE4B, PEL1, PFK, PIM1, PLA2G4A, PLCB1, PML, PNP, PR1, PR1C285, PSM2, PSM2A, PSM4A, PSMB10, PSMB2, PSMB8, PSM9, PSM1, PSM2, PTP11, PTPN1, PR1C285, PSM2, PSM2A, PSM4A, PSMB10, PSMB2, PSMB8, PSM9, PSM1, PSM2, PTP11, PTPN1, PTPN6, RAPGEF6, RBCK1, RIKP1, RIKP2, RNF213, RNF31, RASD2, RTP4, SMD9L1, SAMDI1, SEMP1, SERPING1, SMYF1, SLC2A2, SLC7A2, STC2, SUSD3) or the following signatures, calculated as geometric mean of respective genes: BCLs (BCL2, BCL2L1, MCL1), type I IFN signaling (ADAR, B2M, BAX, B2M, CASP1, CASP8, CCL2, C4D, CD74, CMPK2, CNP, CSF1, CXCL10, CXCL11, DDX60, DHH5X8, EIF2AK2, EIF1, EPSTI1, FM125A, FAM46A, FTSJ1D2, GBP2, GBP4, GMPR, HERC6, HLA-C, IFI27, IFI30, IFI35, IFI44, IFI44L1, IFI41, IFI42, IFI43, IFITM1, IFITM2, IFITM3, IL15, IL18, IRF1, IRF2, IRF7, IRF9, ISG15, ISG20, LAG3, LAMC3, LCP3, LGALS3BP, LYSMD2, MARCH1, METTL7B, MTA2, MTHFD2, MVX, MX1, MX2, MYD88, NAMPT, NCOA3, NFKB1, NFKB1A, NR5C, NMI, NOD1, NUP93, OAS2, OAS3, OASL, OGER, P2Y14, PARP12, PARP14, PDE4B, PEL1, PFK, PIM1, PLA2G4A, PLCB1, PML, PNP, PR1, PR1C285, PSM2, PSM2A, PSM4A, PSMB10, PSMB2, PSMB8, PSM9, PSM1, PSM2, PTP11, PTPN1, PTPN6, RAPGEF6, RBCK1, RIKP1, RIKP2, RNF213, RNF31, RASD2, RTP4, SMD9L1, SAMDI1, SEMP1, SERPING1, SMYF1, SLC2A2, SLC7A2, STC2, SUSD3).

**Results**

**Inhibition of apoptotic caspases does not compromise the therapeutic potential of RT**

To investigate the impact of apoptotic caspases on cell death signaling driven by RT, we subjected wild-type TSA cells to a single RT fraction of 8 Gy or 20 Gy, in the absence or presence of the pan-caspase inhibitor Z-Val-Ala-Asp fluoromethyl ketone (Z-VAD-fmk) and assessed several biomarkers.
of apoptosis, including MOMP and PI uptake (a marker of plasma membrane rupture) 24 and 48 hours post-irradiation. As expected, Z-VD-fmk robustly inhibited MOMP and PI uptake driven at 24 hours by the prototypic apoptosis inducer staurosporine (STS) (Figure 1(a,b)). Consistent with this, short-term exposure of TSA cells to STS for 24 hours resulted in the full proteolytic activation of CASP3 (i.e., generation of the p17 fragment), which could be inhibited by Z-VD-fmk (Figure 1(c)). Irradiated TSA cells began to acquire biochemical markers of apoptosis 48 hours after treatment (Figure 1(d,e)). Accordingly, the Z-VD-fmk-inhibitable generation of the CASP3 p17 fragment became manifest in TSA cells only 48 hours after irradiation (Figure 1(f)). However, Z-VD-fmk did not confer robust cytoprotection in this setting (Figure 1(d,e)), and it failed to affect the clonogenic survival of TSA cells exposed to a single RT fraction of 1 Gy or 3 Gy (Figure 1(g,h)).

Since Z-VD-fmk is poorly specific (it inhibits multiple caspases as well as other proteases) and rather unstable, we decided to harness the CRISPR/Cas technology to generate TSA cell clones lacking the Casp3 gene. Our focus on CASP3 (over other caspases involved in the execution of apoptosis, like CASP6 and CASP7) was dictated by its multipronged immunosuppressive functions, which are not consistently shared by other caspases engaged in the execution of apoptosis, like CASP6 and CASP7.11 Recapitulating data with Z-VD-fmk, Casp3−/− TSA cells were protected from STS-driven rapid apoptosis as compared to their control counterparts (TSA cells subjected to CRISPR/Cas with a non-targeting guide RNA) (Figure 2(a–c)). The Casp3+/− genotype also conferred cytoprotection to TSA cells driven into acute cell death by RT in vitro (Figure 2(d–f)). However, Casp3+/− TSA cells did not differ from their control counterparts in residual clonogenic survival upon irradiation with 1 or 3 Gy (Figure 2(g,h)). Moreover, Casp3+/− TSA cells growing in immunocompetent syngeneic BALB/c mice were more (not less) sensitive to focal irradiation with a single fraction of 20 Gy as compared to their control counterparts, as manifested by improved disease control and increased incidence of tumor eradication (2/10 in control lesions, 4/6 in Casp3+/− lesions) (Figure 2(i)). Altogether, these data demonstrate that CASP3 activation influences the early kinetics of RT-driven cell death (i.e., the speed at which irradiated cells die), but fails to impact on its actual occurrence (i.e., the fraction of cells ultimately succumbing to irradiation), in vitro. Moreover, they indicate that the absence of CASP3 enables superior therapeutic responses to RT in vivo, in immunocompetent syngeneic settings. These findings led us to hypothesize that CASP3 may mediate immunosuppressive effects that limit RT efficacy in vivo.

**Inhibition of apoptotic caspases exacerabtes type I IFN secretion by irradiated cells as it delays the breakdown of cells with cytosolic dsDNA**

We next set to investigate the impact of caspase inhibition on type I IFN release by TSA cells exposed to RT in vitro. Recapitulating published data from the Demaria group,24 TSA cells exposed to a single RT fraction of 8 Gy exhibited increased transcription from the IFNB1 locus, which could be further enhanced by the absence of the Casp3 gene (Figure 3(a)), reminiscent of previous findings with MOMP-inducing chemical agents from the Vaux and Flavell laboratories.21–23 Similar results were obtained by measuring the amount of type I IFN secreted in culture supernatants by ELISA (Figure 3(b)). Surprisingly, Casp3+/− TSA cells also exhibited increased IFNB1 transcription and type I IFN secretion at baseline (Figure 3(a,b)). Type I IFN signaling driven by RT in TSA cells depends on the sensing of cytosolic double-stranded DNA (dsDNA) species by CGAS.24 To test whether accrued type I IFN responses engaged by apoptotic caspase inhibition rely on the same mechanism, we quantified cytosolic dsDNA in caspase-proficient versus caspase-deficient TSA cells maintained in control conditions or exposed to a single RT fraction of 8 Gy. In line with published data,24 RT with 8 Gy efficiently caused the accumulation of dsDNA in the cytosol of irradiated cells (Figure 3(c)). The absence of Casp3 did not only delay the increase in ability of RT to cause the accumulation of dsDNA in the cytosol of TSA cells, but also enabled dsDNA accumulation in non-irradiated cells (Figure 3(c)), reflecting the inability of Casp3+/− TSA cells to produce type I IFN at baseline (Figure 3(a,b)). We interpreted these findings to indicate that apoptotic caspase inhibition promotes the accumulation of cells with cytosolic dsDNA. Consistent with this interpretation, the population of living (PT) Casp3+/− TSA cells detectable 24 hours after RT exhibited lower mitochondrial transmembrane potential as compared to their caspase-proficient counterparts (Figure 3(d)). Of note, this could not be attributed to a general defect in autophagy, which normally ensures the disposal of permeabilized mitochondria.20 Indeed, the starvation-driven lipidation of microtubule associated protein 1 light chain 3 beta (MAP1LC3B) and degradation of sequestosome 1 (SQSTM1, best known as p62), two biochemical markers of autophagy, were not altered by the presence of Z-VD-fmk and in the absence of Casp3 (Suppl. Figure 1).

Altogether, these data suggest that apoptotic caspases inhibit type I IFN secretion by irradiated cells by facilitating the breakdown (and hence complete functional inactivation) of cells undergoing RT-driven MOMP.

**Inhibition of apoptotic caspases exacerbates abscopal responses to RT**

To investigate the impact of apoptotic caspases on abscopal responses, we set to employ control or Casp3+/− TSA cell clones to establish primary tumors in the left flank of immunocompetent, syngeneic BALB/c mice, and control TSA clones to generate slightly asynchronous secondary lesions in the right flank. The therapeutic plan was to treat primary tumors of surface area 15–40 mm² (day 0) with 3 focal RT fractions of 8 Gy each, alone or in combination with systemic inhibition of cytotoxic T lymphocyte associated protein 4 (CTLA4) (Figure 3(e)), which per se is known to have no therapeutic activity in this model.24 However, control TSA clones were highly inefficient at generating secondary lesions when contralaterally implanted two days after Casp3+/− (but not control) clones (Figure 3(f)), which we interpreted as a sign of the ability of Casp3+/− cells to generate some degree of protective immunity, most likely reflecting their capacity to secrete type I IFN in non-stimulated conditions (Figure 3(a–c)). We therefore switched to an alternative experimental plan...
Figure 1. Chemical caspase inhibition fails to impact on the cytotoxic effects of RT.

(a,b) Percentage of TSA cells manifesting apoptosis-associated mitochondrial outer membrane permeabilization and plasma membrane breakdown, as assessed by flow cytometry upon staining with the mitochondrial transmembrane potential-sensitive dye DiOC₆(3) and the vital dye propidium iodide (PI), following 24 hours of culture in control conditions or in the presence of 0.25 μM staurosporine (STS), 20 μM Z-VAD-fmk, or their combination. Representative dotplots (a) and quantitative results (b) are reported. Numbers indicate the percentage of cells in each quadrant. Results are means ± SEM, n = 2–3 independent experiments, ***p < .001 (One-way ANOVA, as compared to untreated cells); ###p < 0.001 (One-way ANOVA, as compared to cells treated with STS).

C. Proteolytic CASP3 activation in TSA cells cultured as in (a,b). Actin, beta (ACTB) levels were monitored to ensure equal loading.

D,E. Percentage of TSA cells manifesting biochemical markers of apoptosis, as assessed by flow cytometry upon staining with DiOC₆(3) and PI 48 hours after γ irradiation with the indicated dose, alone or in presence of 20 μM Z-VAD-fmk. Representative dotplots (d) and quantitative results (e) are reported. Numbers indicate the percentage of cells in each quadrant. Results are means ± SEM, n = 2–3 independent experiments, ***p < .001 (One-way ANOVA, as compared to untreated cells); n.s., not significant (One-way ANOVA, as compared to cells treated with the same dose of radiation only).

F. Proteolytic CASP3 activation in TSA cells cultured as in (d,e). ACTB levels were monitored to ensure equal loading. (g,h). Residual clonogenic potential of TSA cells receiving γ irradiation at the indicated dose, alone or in presence of 20 μM Z-VAD-fmk and allowed to generate colonies for 14 days. Representative images (g) and quantitative results (h) are reported. Results are means ± SEM, n = 2–3 independent experiments, ***p < .001 (One-way ANOVA, as compared to untreated cells); n.s., not significant (One-way ANOVA, as compared to cells treated with the same dose of radiation only). SF, survival fraction.
Figure 2. CASP3 is dispensable for the cytotoxic effects of RT in vitro and in vivo.

(a,b) Percentage of control (SCR) and Casp3−/− TSA cells manifesting apoptosis-associated phosphatidylserine exposure and plasma membrane breakdown, as assessed by flow cytometry upon staining with fluorescent Annexin V (AnnV) and the vital dye propidium iodide (PI), following 24 hours of culture in control conditions or in the presence of 0.1 or 0.25 μM staurosporine (STS). Representative dotplots (a) and quantitative results (b) are reported. Numbers indicate the percentage of cells in each quadrant. Results are means ± SEM, n = 2–3 independent experiments, ***p < .001 (One-way ANOVA, as compared to untreated SCR cells); ###p < .001 (One-way ANOVA, as compared to SCR cells treated with STS).

C. Proteolytic CASP3 activation in SCR and Casp3−/− TSA cells cultured as in A,B. Actin, beta (ACTB) levels were monitored to ensure equal loading.

D,E. Percentage of SCR and Casp3−/− TSA cells manifesting biochemical markers of apoptosis, as assessed by flow cytometry upon staining with fluorescent AnnV and PI 48 hours after γ irradiation with the indicated dose. Representative dotplots (d) and quantitative results (e) are reported. Numbers indicate the percentage of cells in each quadrant. Results are means ± SEM, n = 2–3 independent experiments, ***p < .001 (One-way ANOVA, as compared to untreated SCR cells); n.s., not significant (One-way ANOVA, as compared to SCR cells treated with the same dose of radiation only).

(f) Proteolytic CASP3 activation in SCR and Casp3−/− TSA cells cultured as in (d,e). ACTB levels were monitored to ensure equal loading.

(g,h). Residual clonogenic potential of SCR and Casp3−/− TSA cells receiving γ irradiation at the indicated dose, and allowed to generate colonies for 14 days. Representative images (g) and quantitative results (h) are reported. Results are means ± SEM, n = 2–3 independent experiments, ***p < .001 (One-way ANOVA, as compared to untreated SCR cells); n.s., not significant (One-way ANOVA, as compared to SCR cells treated with the same dose of radiation only). SF, survival fraction.

(i) Growth of SCR and Casp3−/− TSA cells grafted in immunocompetent syngeneic BALB/c mice and optionally subjected to focal γ irradiation with a single fraction of 20 Gy. Results are means ± SEM, n = 6–10 mice/group from 2 independent experiments. Rate of complete disease eradication is reported. ***p < .001 (Two-way ANOVA, as compared to untreated SCR lesions); ###p < .001 (Two-way ANOVA, as compared to irradiated SCR lesions).
**Figure 3.** CASP3 inhibits type I IFN secretion in vitro and abscopal responses to RT in vivo.

(a) IFNβ1 levels in control (SCR) or Casp3−/− TSA cells optionally exposed to γ irradiation (8 Gy) and cultured in control conditions for 48 hours. Results are means ± SEM, n = 2 independent experiments. **p < .01 (One-way ANOVA, as compared to untreated SCR cells); ***p < .001 (One-way ANOVA, as compared to irradiated SCR cells). (b) Type I IFN levels in the supernatant of SCR or Casp3−/− TSA cells optionally exposed to γ irradiation (8 Gy) and cultured in control conditions for 48 hours. Results are means ± SEM, n = 2 independent experiments. **p < .01 (One-way ANOVA, as compared to untreated SCR cells); ***p < .001 (One-way ANOVA, as compared to irradiated SCR cells). (c) Cytosolic DNA accumulation in SCR or Casp3−/− TSA cells optionally exposed to γ irradiation (8 Gy) and cultured in control conditions for 24 hours, as assessed by immunofluorescence with a double-stranded DNA-specific antibody. Representative images (scale bar = 10 μm) and quantitative data are reported. Results are means ± SEM and individual data points, n = 2 independent experiments. **p < .01 (One-way ANOVA, as compared to untreated SCR cells); ***p < .001 (One-way ANOVA, as compared to irradiated SCR cells). (d) Mitochondrial transmembrane potential of control (SCR) or Casp3−/− TSA cells optionally exposed to γ irradiation (8 Gy) and cultured in control conditions for 24 hours, as assessed by flow cytometry upon staining with the mitochondrial transmembrane potential-sensitive dye DiOC6(3) and the exclusion dye propidium iodide (PI). PI+ cells (dead cells) are excluded from the analysis. Representative histograms and quantitative data are reported. Results are means ± SEM, n = 2 independent experiments. ***p < .001 (One-way ANOVA, as compared to irradiated SCR cells); ###p < .001 (One-way ANOVA, as compared to untreated SCR cells). (e) Experimental plans for the assessment of abscopal responses. (f) Rejection rate of SCR TSA lesions implanted in syngeneic, immunocompetent BALB/c mice contralaterally to SCR TSA lesions or TSA lesions from 2 distinct Casp3−/− TSA clones (C1 and C2) established 2 days earlier (as per the original schedule depicted in (e)). Results are from one experiment. (g) Growth of primary (SCR or Casp3−/−, as indicated) and abscopal (SCR) TSA lesions established in syngeneic, immunocompetent BALB/c mice randomly allocated to receive no treatment, focal γ irradiation to the primary lesion in 3 fractions of 8 Gy each, or focal γ irradiation to the primary lesion according to the same schedule plus a CTLA4 blocking antibody (9H10) i.p., as per the revised schedule depicted in (e). Results are means ± SEM, n = 2–10 mice/group from 2 independent experiments. Rate of complete disease eradication at each disease site is reported. **p < .01, ***p < .001, n.s., not significant (Two-way ANOVA, as compared to untreated lesions at the same site).
involving the synchronous establishment of primary and secondary tumors. In this setting, control TSA clones were perfectly able to generate secondary lesions, irrespective of the genotype of contralateral lesions (Figure 3(g)). More importantly, the ability of RT combined with systemic CTLA4 inhibition to control the growth of a non-irradiated, distant lesion was greatly ameliorated by the absence of Casp3 (Figure 3(g)). Moreover, irradiation of Casp3 (but not control) lesions enabled some degree of abscopal control even in the absence of CTLA4 blockade (Figure 3(g)). Thus, CASP3 inhibits abscopal responses driven by RT in mice as it limits type I IFN secretion by irradiated cells.

**Signatures of apoptotic proficiency convey negative prognostic value in breast cancer**

To test the translational value of our findings, we took advantage of METABRIC public database, which provides well-annotated transcriptional data on approximately 2,000 breast tumors and performed survival analyses based on the expression levels of key regulators of apoptosis (Figure 4(a–d)) or broader transcriptional signatures of apoptotic proficiency. Consistent with our preclinical findings, stratifying patients with breast cancer from the METABRIC cohort based on median CASP3 expression levels identified a robust cancer-specific survival advantage for individuals with CASP3Lo (as compared to CASP3Hi) lesions (p = .0016) (Figure 4(a)). In line with the importance of limited CASP3 activation for superior disease outcome in setting, a similar survival advantage could be documented for patients with breast cancers expressing low (as compared to high) levels of apoptotic peptidase activating factor 1 (APAF1), one of the component of the CASP3-activating platform commonly known as apoptosome (p = .0012) (Figure 4(b)), as well as for patients with breast tumors expressing high levels of BCL2 apoptosis regulator (BCL2), BCL2 like 1 (BCL2L1, best known as BCL-XL) and MCL1 apoptosis regulator, BCL2 family member (MCL1), three core inhibitors of MOMP, which we called BCLsHi patients (p < .0001) (Figure 4(c)). Conversely, patients with CASP3Hi breast cancers, which express high levels of another main component of the apoptosome, had superior cancer-specific survival as compared to their CASP3Lo counterparts (p = .0038) (Figure 4(d)), potentially pointing to a positive influence of CASP9 on disease outcome independent of its role in the CASP3 activation pathway. Univariate Cox regression analyses on patients from the METABRIC database exhibiting a signature of type I IFN (Figure 3(g)) or broader transcriptional signatures of apoptotic proficiency in breast cancer patients may influence survival independent of type I IFN signaling and consequent activation of an IFNG-driven immune response. Of note, the top 2 clusters of breast cancer patients identified by the unsupervised clustering of genes differentially expressed by patients subgroups with different apoptotic proficiency, i.e., CASP3Lo versus CASP3Hi, APAFiLo versus APAFiHi, BCLSHi versus BCLSLo, and CASP9Hi versus CASP9Lo, exhibited some (but not complete) overlap with this stratification (Figure 5(a–d)) but invariably had increased prognostic significance (Suppl. Figure 3A–D). Intriguingly, patient clusters defined by differential gene expression based on CASP3, BCLs and CASP9 levels were largely overlapping (Suppl. Figure 3E,F), even though CASP3, BCLs and CASP9 levels exhibited very limited correlation (Suppl. Figure 2).

Driven by these findings, we checked for overlaps in the genes differentially expressed by patients with good versus bad prognosis in the METABRIC database as defined by our four independent signatures of apoptotic proficiency, identifying the following 11 genes: cytochrome P450, family 4, subfamily x, polypeptide 1 (CYP4X1), sushi domain containing 3 (SUSD3), anterior gradient 3, protein disulfide isomerase family member (AGR3), ankyrin repeat domain 30A (ANKRD30A), bone morphogenetic protein receptor, type 1B (BMPR1B), chloride intracellular channel 6 (CLIC6), solute carrier family 7 (cationic amino acid transporter, y + system), member 2 (SLC7A2) and stanniocalcin 2 (STC2), which were all enriched in patients with good prognosis, as well as C-X-C motif chemokine ligand 9 (CXCL9), calmodulin like 5 (CALML5), and lymphotixin B (LTB), which were all enriched in patients with poor prognosis (Figure 6(a,b) and Suppl. Table 3). Strikingly, 7 of these 11 genes have previously been attributed an impact on breast cancer progression and patient survival that is in line with our findings. These genes are AGR3, ANKRD30A, BMPR1B, STC2 and SUSD3, which are all indicators of early disease or good prognosis, as well as CXCL9, CALML5 and SUSD3, which are all enriched in patients with good prognosis and conversely, CYP4X1 (which is our hands was enriched in patients with good survival) has previously been positively correlated with tumor grade.

We therefore harnessed the METABRIC database to test the prognostic value of each of these genes taken individually. As per previous reports, patients with AGR3Hi (p = .0048), ANKRD30AHi (p = .034), BMPR1BHi (p = .046), CALML5Lo (p < .00001), CXCL9Lo (p = .029), STC2Hi (p < .0001), and
SUS3Hi (p < .0001) lesion had improved survival as compared to their Lo or Hi counterparts (Figure 6(c–h) and data not shown). Alongside, we were unable to attribute to CYP4X1 (p = .062) or LTB (p = .74) any prognostic value in this setting. Conversely, we identified a robust survival advantage for both CLIC6Hi (p < .00001) and SLC7A2Hi (p < .00001) patients (Figure 6(c–h)), and neither of these genes had previously been linked to differential survival in patients with breast cancer. Univariate Cox regression analysis confirmed a statistically significant on prognosis for AGR3 (p < .001), ANKRD30A (p = .045), BMPR1B (p = .002), CALML5 (p < .001), CLIC6 (p < .001), SLC7A2 (p < .001), STC2 (p < .001), SUS3D (p < .001), but not for CXCL9 (p = .21), as it suggested a potential effect for CYP4X1.
Unfortunately, such efforts failed to culminate a reflection of defective type I IFN signaling in the tumor microenvironment. As a possibility, these apparently contrasting observations reflect the opposed activity of robust, acute cytotoxic effects against mouse mammary cancer cells and rather sensitize them to RT in vitro and rather than deficiency (but not limited to) myocardial infarction and cerebral stroke.45,46 Unfortunately, such efforts failed to culminate with the approval of cardioprotective or neuroprotective agents based on caspase inhibition for use in humans by American or European regulatory agencies. These rather dismal results mainly reflect (1) the complex architecture of cell death signaling in mammalian cells, whereby the inhibition of single signal transducers generally alters the phenotypic, biological and immunological manifestations of RCD, but fails to convey true cytoprotection;47 and (2) the previously unappreciated multifactorial etiology of similar conditions (which generally involve a large immunological component).46

Here, we demonstrate that the inhibition of CASP3 does not compromise the ability of RT to mediate cytostatic and cytotoxic effects against mouse mammary cancer cells in vivo and rather sensitize them to RT in vitro, in immunocompetent syngeneic settings (Figures 1 and 2). Moreover, CASP3 inhibition exacerbates the capacity of irradiated mammary cancer cells to secrete type I IFN as a consequence of decelerated breakdown of cells with permeabilized mitochondria, and hence favors therapeutically relevant cancer-specific immune responses (Figure 3). To what extent the ability of CASP3 to limit type I IFN secretion in our experimental setting reflected the proteolytic inactivation of CGAS remains to be elucidated.

Of note, multiple signatures of apoptotic proficiency (rather than deficiency) turned out to be associated with poor disease outcome in patients with breast cancer from the METABRIC database (Figure 4). However, that was not a reflection of defective type I IFN signaling in the tumor microenvironment, as a genetic signature of type I IFN signaling was associated with inferior (not superior) survival (Figure 4). As a possibility, these apparently contrasting observations reflect the opposed activity of robust, acute versus indolent, chronic type I IFN signaling in the breast microenvironment.49

Differential gene expression analysis of patient subgroups with good versus bad survival (based on 4 independent signatures of apoptotic proficiency) identified 11 genes similarly enriched in either of these groups, 8 of which had previously been attributed an impact on breast cancer progression or patient survival (Figures 5 and 6).34,37-43 The expression levels of these genes also conveyed prognostic value in patients from

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### Table 1. Univariate Cox proportional-hazard regression analysis.

| Variable                      | HR   | 95% CI    | p value |
|-------------------------------|------|-----------|---------|
| Age                           | 1.02 | 1.02–1.03 | <0.001 |
| Stage                         | 2.08 | 1.82–2.39 | <0.001 |
| Stage II vs. I                | 2.08 | 1.66–2.60 | <0.001 |
| Stage III vs. I               | 4.57 | 3.36–6.22 | <0.001 |
| Stage IV vs. I                | 7.26 | 2.53–14.92| <0.001 |
| Chemotherapy                  |      |           |         |
| YES vs. NO                    | 1.61 | 1.35–1.91 | <0.001 |
| Hormone therapy               |      |           |         |
| YES vs. NO                    | 1.09 | 0.93–1.28 | 0.29   |
| Radiotherapy                  |      |           |         |
| YES vs. NO                    | 0.89 | 0.76–1.04 | 0.14   |
| Grade                        | 1.59 | 1.39–1.83 | <0.001 |
| 2 vs. 1                      | 1.82 | 1.20–2.76 | <0.001 |
| 3 vs. 1                      | 2.8  | 1.87–4.20 | <0.001 |
| Tumor size                    | 2.27 | 1.93–2.67 | <0.001 |
| ER status                     |      |           |         |
| Positive vs. Negative         | 0.68 | 0.57–0.81 | <0.001 |
| PR status                     |      |           |         |
| Positive vs. Negative         | 1.74 | 1.43–2.14 | <0.001 |
| HER2 status                   |      |           |         |
| Gain                          | 0.69 | 0.47–1.01 | 0.056  |
| Neutral vs. Gain              | 0.67 | 0.56–0.80 | <0.001 |
| Undefined vs. Gain            | 1.25 | 0.51–3.04 | 0.75   |
| Oncotree Code                 |      |           |         |
| Invasive ductal carcinoma (IDC) vs. Breast | 2.67 | 0.86–8.29 | 0.09   |
| Invasive lobular carcinoma (ILC) vs. Breast | 2.59 | 0.80–8.32 | 0.11   |
| Invasive mixed mucinous carcinoma (IMMC) vs. Breast | 1.4 | 0.31–6.27 | 0.65   |
| Histological subtype          |      |           |         |
| Lobular vs. Ductal/No Special Type | 0.95 | 0.70–1.28 | 0.72   |
| Medullary vs. Ductal/No Special Type | 0.77 | 0.40–1.49 | 0.44   |
| Mixed vs. Ductal/No Special Type | 0.93 | 0.71–1.70 | 0.57   |
| Mucinous vs. Ductal/No Special Type | 0.51 | 0.19–1.37 | 0.18   |
| Tubular/circriform vs. Ductal/No Special Type | 0.09 | 0.01–0.67 | 0.018  |
| Other vs. Ductal/No Special Type | 0.37 | 0.12–1.14 | 0.08   |
| Inferred menopausal state     |      |           |         |
| Pre- vs. Post-                | 0.78 | 0.65–0.94 | 0.009  |
| Genes and gene signatures (Hi vs. Lo) |     |           |         |
| AGR3                          | 0.94 | 0.91–0.98 | <0.001 |
| ANKRD23A                      | 0.97 | 0.94–1.00 | 0.045  |
| APAF1                         | 1.23 | 0.96–1.57 | 0.1    |
| BCLs                          | 0.53 | 0.43–0.64 | <0.001 |
| BMPR1B                        | 0.62 | 0.43–0.91 | 0.02   |
| CALMILS                       | 2.61 | 1.82–3.69 | <0.001 |
| CASP3                         | 1.38 | 1.14–1.68 | <0.001 |
| CASP9                         | 0.61 | 0.42–0.90 | 0.011  |
| CIC6                          | 0.32 | 0.22–0.46 | <0.001 |
| CXCL9                         | 1.03 | 0.35–8.18 | 0.21   |
| CYP4F1                        | 0.96 | 0.92–1.01 | 0.032  |
| FGF signaling signature       | 1.08 | 0.85–1.36 | 0.54   |
| LTB                           | 0.75 | 0.43–1.29 | 0.3    |
| SLC7A2                        | 0.88 | 0.84–0.91 | <0.001 |
| STC2                          | 0.89 | 0.86–0.99 | <0.001 |
| SUSD3                         | 0.83 | 0.78–0.87 | <0.001 |
| Type I IFN signaling signature| 1.16 | 0.99–1.36 | 0.066  |

**Abbreviations:** CI, confidence interval; HR, hazard ratio.

### Table 2. Multivariate Cox proportional-hazard regression analysis.

| Variable                      | HR   | 95% CI    | p value |
|-------------------------------|------|-----------|---------|
| Age                           | 1.03 | 1.02–1.04 | <0.001 |
| Stage                         | 1.48 | 1.24–1.77 | <0.001 |
| Tumor size                    | 1.67 | 1.32–2.11 | <0.001 |
| HER2 status                   |      |           |         |
| Positive vs. Negative         | 1.47 | 1.15–1.90 | 0.002  |
| Inferred menopausal state     |      |           |         |
| Pre- vs. Post-                | 0.64 | 0.46–0.88 | 0.0074 |
| Genes (Hi vs. Lo)             |      |           |         |
| CALMILS                       | 1.82 | 1.21–2.88 | 0.0043 |
| SLC7A2                        | 0.94 | 0.89–0.99 | 0.033  |
| SUSD3                         | 0.91 | 0.85–0.98 | 0.012  |

**Abbreviations:** CI, confidence interval; HR, hazard ratio.
the METABRIC dataset (Figure 6 and Tables 1 and 2), with the potential exception of CYP4X1. Of the remaining three genes, LTB failed to impact cancer-specific overall survival in the METABRIC cohort (Tables 1 and 2), while both CLIC6 and SLC7A2 were strongly associated with improved disease outcome (Figure 6 and Tables 1 and 2), although only SLC7A2 as
an independent prognostic biomarker (Table 2). The molecular mechanisms whereby CLIC6 expression may positively impact on survival remain obscure. Conversely, SLC7A2 (which was enriched in patients with good prognosis) is known for its anti-inflammatory effects, at least in part linked to its key role in the immunosuppressive activity of myeloid-derived suppressor cells. These observations lend further support to the notion that chronic, indolent
inflammation in the breast microenvironment may support disease progression.

With the unavoidable limitations imposed by retrospective studies on large (and hence heterogeneous) public databases, our findings may suggest that apoptotic proficiency influences the survival of breast cancer patients via pathways that are not necessarily linked to type I IFN signaling. Thus, caspase inhibitors may constitute a promising partner for RT for breast cancer patients or subsets thereof. Although no caspase inhibitor is currently available for clinical use, the investigational agent emricasan has been granted Fast Track designation by the US Food and Drug Administration (FDA) in early 2016 for the treatment of liver cirrhosis caused by nonalcoholic steatohepatitis, and recent data from randomized trials confirm preliminary observations indicating that emricasan is safe and well tolerated, at least in this non-oncological setting. Whether emricasan can be safely and efficiently combined with RT for the treatment of breast cancer remains an unexplored possibility.

Disclosures
LG provides remunerated consulting to OmniSEQ (Buffalo, NY, USA), Astra Zeneca (Gaithersburg, MD, USA), Inzen (New York, NY, USA) and the Luke Heller TECPR2 Foundation (Boston, MA, USA), and he is member of the Scientific Advisory Committee of OmniSEQ (Buffalo, NY, USA).

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