CD95L cell surface cleavage triggers a pro-metastatic signaling pathway in triple negative breast cancer.

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Abstract

Triple-negative breast cancers (TNBC) lacking estrogen and progesterone receptors and HER2 amplification have a relatively high risk of metastatic dissemination, but the mechanistic basis for this risk is not understood. Here we report that serum levels of CD95L are higher in TNBC patients compared to other breast cancer patients. Metalloprotease-mediated cleavage of CD95L expressed by endothelial cells surrounding tumors generates a gradient that promotes cell motility, due to formation of an unconventional CD95-containing receptosome termed the motility-inducing signaling complex. Formation of this complex was instrumental for Nox3-driven ROS generation. Mechanistic investigations revealed a Yes-Orai1-EGFR-PI3K pathway that triggered migration of TNBC cells exposed to CD95L. Our findings establish a pro-metastatic function for metalloprotease-cleaved CD95L in TNBCs, revisiting its role in carcinogenesis.
Introduction

Human breast tumors are heterogeneous, both in their clinical expression and their molecular profiles. Triple-negative breast cancers (TNBCs) are characterized by negative immunohistochemical staining for estrogen and progesterone receptors and human epidermal growth factor receptor-2 (HER2), and represent 10–20% of all breast cancers. TNBCs account for a disproportionate number of patient early deaths due to their aggressive nature and the lack of effective therapeutic treatment options (1). Gaining insight into the molecular mechanism(s) that promote TNBC invasiveness could be of significant value for the design of treatment strategies aimed at preventing metastatic dissemination of these cancers. In addition, prognostic markers are needed to discriminate among TNBC patients those who are at highest risk for metastatic relapse.

CD95 ligand (CD95L, also known as FasL) belongs to the TNF (Tumor Necrosis Factor) family and is the ligand for the “death receptor” CD95 (Fas/APO1). CD95L is a transmembrane “cytokine” whose extracellular domain can be cleaved by metalloproteases (2), to produce a soluble ligand. This soluble form was initially described as an inert ligand that competes with its membrane-bound counterpart for binding to CD95, thus acting as an antagonist of the death signal (3, 4). More recent findings have shown that metalloprotease-cleaved-CD95L (cl-CD95L) can actively participate in aggravating inflammation in chronic inflammatory disorders, such as systemic lupus erythematosus (5, 6) and may exert pro-oncogenic functions by promoting the survival of ovarian and liver cancers (7) and chemotherapy resistance of lung cancers (8) through molecular mechanisms that remain to be elucidated.

Binding of transmembrane CD95L to CD95 leads to the recruitment of the adaptor protein Fas-associated death domain protein (FADD) to the intracellular region of CD95 called
the death domain (DD). In turn, FADD binds to caspases 8 and 10. This CD95/FADD/caspase complex is known as the Death-Inducing Signaling Complex (DISC) (9) and plays a pivotal role in the initiation of the apoptotic signal. By contrast, cl-CD95L fails to induce DISC formation and instead promotes the formation of an atypical receptosome that we have designated Motility-Inducing Signaling Complex (MISC) (6). MISC formation leads to the induction of the pro-oncogenic phosphoinositide 3-kinase (PI3K) signaling pathway (6, 10) through a molecular mechanism that remains to be elucidated.

Here, we show that the level of cl-CD95L is higher in the blood of patients with TNBC than in that of non-TNBC patients and is associated with increased risk of developing distant metastases. Moreover, we demonstrate that after cleavage by a metalloprotease, the soluble CD95L promotes the motility of TNBC cells by inducing nicotinamide adenine dinucleotide phosphate-oxidase oxidase-3 (Nox3)-driven ROS generation, which activates Src kinase c-yes leading to PI3K signaling through activation of the EGF receptor in an EGF-independent manner.
Materials and Methods

Ethics Statement.

All clinical investigation was conducted in accordance with the principles outlined in the Declaration of Helsinki.

Cell lines, antibodies, plasmids and other reagents.

Human breast adenocarcinoma cells MDA-MB-231, MDA-MB-468 and Hs578T were maintained in DMEM supplemented with 8% (v/v) heat-inactivated FCS and 2 mM L-glutamine at 37°C in a 5% CO2 incubator. Silencing experiments were performed by lentiviral transduction of TNBC cells using shRNAmir-pGIPZ vectors (OpenBiosystems, Waltham, MA, USA). All reagents are described in supplementary methods.

Mouse experiments.

NOD/SCID/γc null mice (NSG) were obtained from Dr C. Rivers (UK). Luciferase-expressing MDA-MB-231 cells resuspended with or without cl-CD95L (100 ng/ml) (5×10⁵ cells in 50μL PBS/Matrigel) were transplanted into mammary fat pads of mice (7 weeks old female). Next, caudal vein injections of cl-CD95L (10 μg/kg) or control medium were repeated 5 days a week until day 34. Bioluminescence analysis was performed using PhotonIMAGER (Biospace Lab), following intraperitoneal injection of luciferin (30mg/kg). Tumor volume was calculated using the formula V = 0.52 (L×W²). After completion of the analysis, autopsy of mice was done, and organ luminescence was assessed.
DISC/MISC analysis.

TNBC cells (3×10^7 cells) were incubated with 100 ng/ml of cl-CD95L (MISC) or APO1-3 (DISC) for the indicated times. The cells were lysed, 1 μg of Apo1-3 was added to the cell lysate and CD95 was immunoprecipitated by the addition of protein A-sepharose beads (Sigma). For the immunoprecipitation of EGFR, 1 μg of anti-EGFR (Santa Cruz Biotechnology, CA, USA) was added to the cell lysate and EGFR was immunoprecipitated with protein A-sepharose beads. After extensive washing, the immune complex was resolved by SDS-PAGE.

Ca^{2+} monitoring.

Ca^{2+} monitoring was performed as previously described (11).

Statistical analyses and Supplementary methods are available on-line.
Results

Serum CD95L level predicts metastasis in breast cancers.

Although high CD95L levels have been detected by immunohistochemistry in breast cancer tissue (12), the role of this ligand in carcinogenesis remains unknown. To address this question, serum CD95L levels were measured in women with breast cancer. Blood dosages showed higher CD95L levels in women affected by TNBC than in non-TNBC patients (98.94±45.37, n=39 vs 52.79±26.2, n=103, P<0.0001) and subjects with benign breast diseases (98.94±45.37, n=39 vs 30.04±28.52, n=8, P<0.0001, Fig. 1A). Of note, the quantity of CD95L exhibited a heterogeneous distribution in non-TNBC patients (Fig.1A); however, when this cohort of patients was sub-divided based on the occurrence of relapse, CD95L levels were significantly higher in relapsing patients than in those without relapse (62.72±31.31, n=39 vs 47.68±21.77, n=64, P=0.0053, Fig. 1A). In addition, Kaplan-Meier analyses revealed that both TNBC and non-TNBC patients with CD95L concentrations ≥80 pg/mL had significantly reduced disease-free survival (Fig.1B) and an increased occurrence of metastases (Fig.1C). This predisposition to metastasis was even more pronounced in patients with serum CD95L concentrations ≥120 pg/mL (Fig.1C). To further investigate if cl-CD95L is endowed with a pro-metastatic role toward TNBC cells, we orthotopically transplanted luminescent TNBC cells (MDA-MB-231) in NOD/SCID/γc mice and monitored both tumor growth and metastatic dissemination with or without repeated injections of purified cl-CD95L. Of note, injection of cl-CD95L did not modify the tumor volume (Fig.S1A), while as observed by bioluminescence imaging, it augmented the spread of TNBC cells, which seemed to metastasize to the lungs (Fig.1D). To confirm this observation, mice were sacrificed, and luminescence was assessed in different organs (Fig.1E). Although no luminescence was detected in hearts and livers of control
and cl-CD95L-injected mice (Fig.S1B), lungs and brachial draining lymph nodes experienced a dramatic TNBC cell invasion in mice injected with cl-CD95L as compared to control mice (Fig.1E). Overall, these findings indicated that high amounts of soluble CD95L mainly detected in TNBC women may participate in metastatic progression.

Next, to identify cells responsible for CD95L expression in breast cancer patients, immunohistochemistry was performed in breast tissue sections from healthy, non-TNBC and TNBC patients (Fig.1F). CD95L staining was undetectable in healthy subjects, whereas breast tissues from non-TNBC and TNBC patients exhibited a level of CD95L expression correlated with serum concentrations (Fig.1F). Indeed, CD95L was expressed in an increasing gradient from healthy subjects to TNBC patients (Fig.1F). Furthermore, CD95L was expressed in endothelial cells of blood vessels (CD31+) surrounding tumor masses but not in the lymphatic endothelium (D2-40+) (Fig.1G). These findings indicated that CD95L is ectopically expressed in tumor blood vessels and its cleavage by metalloprotease releases a poor prognostic marker associated with metastatic dissemination in TNBC patients.

Cleaved CD95L induces a p110β and Ca²⁺-driven pro-motile signal in TNBC cells.

Given that CD95L stems from blood vessels surrounding breast cancer cells and is associated with the risk of metastasis, we next wondered whether this ligand contributed to TNBC intravasation by promoting cell motility. Different TNBC cell lines exposed to cl-CD95L showed a dramatic increase in their motility (Fig.2A). Exposure to amounts of CD95L equal to those measured in breast cancer patients was sufficient to trigger cell motility. Indeed, titration of cl-CD95L showed that cl-CD95L at 100 pg/mL, a dose corresponding to the mean concentration measured in TNBC patients (98.94±45.37 pg/mL; Fig.1A), induced migration of TNBC cells
(Fig. S1C). To confirm the effect of soluble CD95L on promoting the motility of breast cancer cells, we incubated TNBC cells in presence of sera from healthy donors or TNBC patients and evaluated cell migration using Boyden chambers. The results showed that the sera from women with TNBC, unlike that from healthy subjects, contained a molecule that enhanced the migration of breast cancer cells (Fig. 2B). This pro-motile factor was identified as soluble CD95L because pre-incubation of TNBC sera with a neutralizing anti-CD95L antibody abrogated their pro-motile effect (Fig. 2B).

Activation of the PI3K signaling pathway promotes different steps leading to metastasis including cell motility and intravasation/extravasation (13). PI3Ks are lipid kinases generating the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which serves as a docking site for various signaling factors. For instance, binding of the serine-threonine kinase Akt to PIP3 leads to its redistribution to the plasma membrane, where it undergoes phosphorylation and activation at Ser473 (For review, see (14)). In all tested TNBC cells, cl-CD95L induced rapid phosphorylation of Akt at Ser473 (Fig. S2A). Furthermore, inhibition of the PI3K signal using the pharmacologic inhibitor LY294002 abrogated the migration of TNBC cells exposed to cl-CD95L (Fig. S2B), indicating that after cleavage by metalloprotease, this ligand acts as a chemoattractant for malignant breast cells through activation of PI3K. Class I PI3Ks comprise four catalytic isoforms (α, β, δ and γ). P110α and β are ubiquitously expressed, whereas p110δ and γ expression are restricted to hematologic cells. To evaluate the contribution of p110α and β in CD95-mediated cell motility in TNBC cells, we analyzed the effect of pharmacological inhibitors of p110α and p110β (Fig. S2C). The p110β inhibitor TGX-221 was much more efficient than a drug targeting p110 α (PIK-90) to prevent both Akt phosphorylation (Fig. S2D) and cell migration (Fig. S2E) in TNBC cells exposed to cl-CD95L. To confirm these
observations, RNA interference was used to silence expression of p110α or p110β isoform (Fig.S2F). Whereas down-regulation of p110α did not alter CD95-mediated PI3K activation (Fig.2C) and cell migration (Fig.2D), silencing of p110β inhibited Akt phosphorylation (Fig.2C) and cell motility (Fig.2D). These findings demonstrated that cl-CD95L selectively activates the PI3K catalytic subunit p110β, which promotes cell migration in TNBC cells.

**Cl-CD95L triggers MISC formation in TNBC cells.**

To decipher the initial events leading to p110β activation, TNBC cell lines were stimulated with cl-CD95L followed by immunoprecipitation of CD95. Analysis of the resulting immune complex revealed that CD95 did not bind to FADD and caspase-8 but did recruit c-yes (Fig.2E). In addition, down-regulation of c-yes expression (Fig.S2G) prevented both Akt phosphorylation (Fig.S2H) and cell migration (Fig.2F) in TNBC cells exposed to cl-CD95L.

Calcium ions (Ca$^{2+}$) participate in cell signaling as a second messenger that relies on intensity (cytosolic concentration), temporal parameters (*i.e.*, duration and frequency) and spatial localization to trigger a variety of cellular responses. Because Ca$^{2+}$ plays a pivotal role in cell motility (15), we analyzed its impact on the signaling pathway induced by cl-CD95 in breast cancer cells. In nonexcitable cells, Ca$^{2+}$ responses mainly occur through a biphasic signal caused by activation of inositol 1,4,5-trisphosphate (IP$_3$) receptors and the release of Ca$^{2+}$ from the endoplasmic reticulum (ER) followed by a sustained Ca$^{2+}$ entry across the plasma membrane (16). Recently, STIM1 was identified as the ER-located Ca$^{2+}$ sensor that links ER depletion to activation of the plasma membrane Ca$^{2+}$ channel formed by Orai1 subunits, allowing Ca$^{2+}$ to enter the cell (17). This store-operated Ca$^{2+}$ (SOC) entry plays pivotal roles in both the replenishment of the ER store and cell signaling (18). Pre-treatment of breast cancer cells with
the Ca\textsuperscript{2+} chelator, BAPTA-AM or the pharmacological inhibitor of SOC channels BTP2 inhibited both CD95-mediated PI3K activation and cell migration (Fig.3A-B). To determine whether Orai1 participated in CD95 signaling in TNBC cells, Orai1 expression was blocked using shRNA mir. As shown in Figure 3C, down-regulation of Orai1 did not alter the CD95-mediated Ca\textsuperscript{2+} mobilization from the ER, but it abolished the subsequent Ca\textsuperscript{2+} entry observed in TNBC cells stimulated with cl-CD95L. Of note, Orai1-driven Ca\textsuperscript{2+} entry was essential for inducing PI3K activation and cell migration (Fig.3D-E). To further characterize the mechanistic link between CD95 engagement by cl-CD95 and Ca\textsuperscript{2+} signaling in breast tumor cells, the contribution of c-yes and p110\(\beta\) to the CD95-mediated Ca\textsuperscript{2+} response was examined. Silencing of c-yes abrogated CD95-mediated Ca\textsuperscript{2+} signaling (Fig.3F). On the other hand, although Ca\textsuperscript{2+} signaling contributed to PI3K activation (Fig.3A), the opposite was not true, as p110\(\beta\) knock-down did not affect the CD95-mediated Ca\textsuperscript{2+} response (Fig.3F). These results indicated that c-yes occupies a proximal position in the sequence of events leading to the activation of the Ca\textsuperscript{2+}/PI3K signaling pathway in breast tumor cells exposed to cl-CD95L.

**ROS production initiates the CD95-mediated non-apoptotic signal.**

Because recent reports have shown that Src kinases can behave as redox sensors promoting cell migration in leukocytes (19), we investigated whether CD95 activates c-yes through the generation of ROS. Different TNBC cells exposed to cl-CD95L experienced a rapid increase in intracellular ROS (Fig.S3A), which was blocked by pre-treatment with reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPHox) inhibitors, such as DPI and apocynin (Fig.4A). To determine the effect of ROS on c-yes activation, c-yes was immunoprecipitated and its auto-phosphorylation status (hallmark of its activation) was
evaluated by immunoblotting using a phospho-Src family antibody, recognizing src when phosphorylated at tyrosine 416 and c-yes at an equivalent site (tyrosine 426). In the presence of cl-CD95L, TNBC cells undergo rapid activation of c-yes that is inhibited by NADPHox inhibitors (Fig.4B and Fig.S3B). Also, pre-treatment of mammary cancer cells with DPI or apocynin inhibited CD95-mediated activation of Akt (Fig.S3C-D) and cell migration (Fig.S3E). The Nox family is defined by seven distinct catalytic isoforms, namely Nox-1 to -5 and Duox-1 and Duox-2. While Nox-1, -2, -3, and -4 are associated with p22Phox, which is involved in their proper membrane targeting and activity, Nox-5, Duox-1, and Duox-2 do not require p22Phox for their activity (20). MISC analysis showed that p22Phox was recruited after stimulation with cl-CD95L (Fig.4C). In addition, down-regulation of p22Phox (Fig.S3F) prevented activation of c-yes and Akt (Fig.4D) and cell migration in TNBC cells exposed to cl-CD95L (Fig. 4E and S3G), supporting that association of p22Phox with Nox-1, -2, -3 or -4 orchestrated the CD95-mediated ROS production. To identify the Nox subunit responsible for ROS generation in TNBC cells stimulated with cl-CD95L, we next analyzed the recruitment of Nox-1, -2, -3, or -4 to the MISC. As shown in Figure 4F, only Nox-3 was recruited in the MISC of TNBC cells stimulated with cl-CD95L. In addition, while Nox-2 down-regulation (Fig.S3H) did not alter cell migration in cl-CD95L-stimulated TNBC cells (Fig.S3I), Nox-3 silencing (Fig.S3H) completely abrogated it (Fig.S3I). Finally, pharmacological and genetic inhibitions of NADPH oxidase completely abrogated the CD95-mediated Ca^{2+} response (Fig.S3J), confirming that CD95-triggered ROS generation is a proximal event in the signaling cascade induced by metalloprotease-cleaved CD95L in TNBC cells. These findings highlighted that TNBC cells exposed to cl-CD95L undergo a rapid Nox3-driven ROS production resulting in the activation of c-yes and its downstream signaling pathway.
**EGFR is essential for cl-CD95L-mediated migration of breast cancer cells.**

Taken together, these findings raise the question of how PI3K can be activated in TNBC cells in the presence of cl-CD95L. An *in silico* analysis of short linear motifs (21) in the intracellular region of CD95 did not reveal any consensus sequences that could account for recruitment of p110β or its regulatory subunit p85 after “death receptor” phosphorylation by c-yes, suggesting that at least one additional factor connects CD95 to PI3K signaling. Src kinase family has been reported to phosphorylate EGFR at Tyr845, a modification that stabilizes the activation loop and maintains the receptor in an active state (22). Moreover, src kinase can also associate with EGFR to form a heterocomplex in TNBC cells (23). Because, 72% of TNBC cells express EGFR (24), and its expression is correlated with cell migration (25), we examined the potential contribution of EGFR to CD95 signaling. EGFR was expressed predominantly in TNBC cell lines compared to non-TNBC cells (Fig.S4A-B). Based on our immunoprecipitation experiments showing an interaction between CD95 and c-yes (Fig.2E), we hypothesized that recruitment of c-yes to MISC may lead to EGFR activation, which in turn may serve as a molecular platform to elicit the PI3K (p110β) signaling pathway. To explore this hypothesis, the phosphorylation status of EGFR at Tyr845 was monitored. As shown in Figure 5A, exposure of MDA-MB-468 breast cancer cells to cl-CD95L resulted in phosphorylation of EGFR at Tyr845, which reached a peak at 2 min and disappeared after 10 min of stimulation. This EGFR phosphorylation preceded Akt activation (Fig.5A). EGFR activation relied on CD95 stimulation because pre-incubation of TNBC cells with neutralizing anti-CD95L antibody (Nok-1) totally abrogated EGFR phosphorylation (Fig.S4C). In addition, EGFR phosphorylation was dependent on c-yes, as shown by the loss of EGFR phosphorylation in TNBC cells in which c-yes
expression was suppressed (Fig.5B). Next, we used biochemical and imaging approaches to elucidate whether EGFR was recruited to the MISC in TNBC cells exposed to cl-CD95L. Immunoprecipitation of CD95 or EGFR from TNBC cells exposed to cl-CD95L revealed the rapid formation of an immune complex containing CD95/c-yes/EGFR/p110β (Fig.5C-D). These biochemical observations were confirmed by the co-localization of EGFR and CD95 at the leading edge of emitted pseudopodia in TNBC cells (Fig.S4D-E). Overall, these findings demonstrated that EGFR is recruited to the MISC in TNBC cells exposed to cl-CD95L. To further investigate the role of EGFR in the CD95-mediated cell motility, we used the EGFR inhibitor erlotinib. Erlotinib prevented CD95-mediated Akt phosphorylation in TNBC cells (Fig.S5A-B). In addition, erlotinib abolished migration of TNBC cells exposed to cl-CD95L (Fig.S5C). To prove that EGFR was instrumental in CD95-mediated cell signaling, the tyrosine kinase receptor was next silenced (Fig. S5D). Down-regulation of EGFR expression inhibited both CD95-mediated PI3K activation (Fig.5E) and cell migration in TNBC cells (Fig.5F and Fig.S5E). Examination of the role of EGFR in the cl-CD95L-induced Ca^{2+} response showed that although the binding of EGF to EGFR evoked a Ca^{2+} response, which was blocked by erlotinib (Fig.S5F), neither treatment with erlotinib nor EGFR silencing altered the CD95-mediated Ca^{2+} response (Fig.S5F). This latter observation questioned the involvement of EGF in the recruitment and activation of EGFR in breast cancer cells stimulated with cl-CD95L. To address the possible involvement of EGF in this process, cl-CD95L-mediated signaling was examined in TNBC cells pre-incubated with cetuximab, an antibody that binds to the EGFR ectodomain and prevents its interaction with EGF (26). Although this blocking antibody abrogated the EGF-induced Ca^{2+} response, it did not affect CD95-mediated Akt activation (Fig.S5G) and cell motility (Fig.S5H),
supporting the notion that CD95-driven recruitment of EGFR occurs through an EGF-independent mechanism in TNBC cells.
Discussion

The results of the present study show that an elevated serum concentration of CD95L in women diagnosed with breast cancer is associated with poor prognosis and high risk of distant metastasis. Furthermore, the distribution of CD95L on endothelial cells of blood vessels suggests that its shedding by a yet unknown metalloprotease may create the concentration gradient required to promote intravasation of breast tumor cells and thus distant cancer metastases.

Further analysis of the molecular pathway connecting the “death receptor” CD95 to downstream PI3K signaling in TNBC cells exposed to cl-CD95L showed the recruitment and activation of EGFR. Although EGFR was necessary for CD95-mediated activation of PI3K, it was dispensable in the Ca\(^{2+}\) response, indicating that the two pathways diverge early in the unconventional pathway of cl-CD95L-triggered signaling. This divergence must occur downstream of ROS generation and subsequent c-yes activation and upstream of EGFR activation, as inhibition of NADPH oxidase and silencing of c-yes expression abolished both PI3K activation and Ca\(^{2+}\) signaling in TNBC cells exposed to cl-CD95L. In the presence of EGF, TNBC cells evoked a Ca\(^{2+}\) response that was abolished by pre-treatment with the tyrosine kinase inhibitor erlotinib. Given that erlotinib and EGFR down-regulation did not alter the Ca\(^{2+}\) signal observed in breast cancer cells exposed to cl-CD95L, we surmise that recruitment and activation of EGFR by CD95 occurs through an atypical mechanism independent of EGF. Confirming this assumption, the EGFR neutralizing antibody cetuximab did not impair CD95-driven EGFR activation in breast cancer cells. Conventionally, binding of EGF ligands to EGFR leads to receptor dimerization, activation of its intrinsic tyrosine kinase activity and subsequent phosphorylation of downstream signaling molecules. However, this dogma has been challenged...
by the discovery that the activation of EGFR-mediated signaling can occur in a ligand-independent manner in the presence of ROS (27). In addition, G protein-coupled receptor activation can mediate EGFR transactivation through src family tyrosine kinases (28), and c-src itself is able to facilitate EGFR activation by phosphorylation of Tyr^{845} (29). Our results bring to light a novel mechanism for EGFR activation in TNBC cells stimulated with metalloprotease-cleaved CD95L. From a mechanistic standpoint, CD95-driven EGFR activation is dependent on the generation of ROS by Nox-3. ROS activate c-yes, which in turn recruits and activates EGFR.

Similar to EGFR, c-Met is a transmembrane receptor tyrosine kinase for hepatocyte growth factor (HGF). Recent reports have shown that the CD95 signaling pathway can be modulated by c-Met (30). Indeed, the amino-acid residues YLGA in c-Met can bind CD95 (30) and prevent its homotrimeric self-aggregation, a pivotal step in the implementation of the apoptotic signal (31). It is noteworthy that the YLGA motif is not detected in the EGFR sequence, and while the CD95/c-Met association is lost when CD95L interacts with its receptor (32), binding of cl-CD95 is mandatory for recruitment of EGFR by CD95. Taken together, these findings strongly suggest that receptor tyrosine kinases such as c-Met and EGFR can interact with CD95 by different molecular mechanisms. More importantly, these results indicate that implementation of CD95-mediated non-apoptotic signals may occur through the recruitment of tyrosine kinase receptors that behave as docking sites for several proteins, including PI3K.

Because serum CD95L level is associated with relapse in breast cancers, exhaustive identification of the components of the MISC and characterization of the molecular pathway leading to the induction of CD95-mediated non-apoptotic signaling are critical to understand how this “death receptor” can transmit non-death signals depending on the ligand with which it interacts. Our findings point to the activation of the src kinase c-yes, which implements Orai1-
mediated store-operated calcium (Ca\(^{2+}\)) entry (SOCE), recruits EGFR, and induces the downstream activation of the p110\(\beta\) catalytic isoform of PI3K (summarized in Fig.6).

In summary, this study identified serum CD95L level as a new prognostic marker of metastatic dissemination in women with breast cancer. This finding may help to guide clinicians in the selection of the most appropriate treatment regimen for patients with high levels of cl-CD95L who should undergo intensive therapy, while patients with low cl-CD95L should be spared. In addition, the elucidation of this atypical CD95-mediated signaling pathway provides new therapeutic targets for preventing metastatic dissemination in TNBC. Although inhibitors of EGFR kinase activity may be attractive therapeutic agents, especially because gefitinib and erlotinib are already in use for the treatment of non-small cell lung cancer, most patients on prolonged gefitinib and erlotinib treatment develop secondary mutations in the EGFR kinase domain that block drug binding, leading to clinical resistance (33, 34). Therefore, inhibition of the CD95/CD95L interaction may be a more appropriate treatment approach, especially because such an inhibitor already exists and is well-tolerated by patients (35).
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Figure Legends

Figure 1. Serum CD95L is increased in TNBC women and associated with metastatic dissemination.

A. Serum CD95L in patients diagnosed with TNBC or non-TNBC patients was measured by ELISA (n indicates the number of patients per group). *** and ** indicate P < 0.0001 and 0.01 by a two-tailed Student’s t test. B. Kaplan-Meier relapse-free survival curves comparing patients with CD95L concentrations higher (thick line) or lower (dotted line) than 80 pg/mL. C. Left panel: Kaplan-Meier analysis of distant metastasis in patients with CD95L concentrations higher (thick line) or lower (dotted line) than 80 pg/mL. Right panel: Kaplan-Meier analysis of distant metastasis in patients with CD95L concentrations higher (thick line) or lower (dotted line) than 120 pg/mL. D. Luciferase-expressing MDA-MB-231 cells were orthotopically injected in NOD/SCID/γc null mice. Then, cl-CD95L (10 μg/kg) or vehicle was repeatedly injected and metastatic dissemination of TNBC cells was evaluated by bioluminescence imaging (BLI) at day 21. Black arrows depict mice showing luciferase-expressing MDA-MB-231 spreading. E. Mice treated as depicted in D were sacrificed at day 34. Intensity of luminescence was quantified in indicated organs. * and ** indicate p<0.05 and 0.01 calculated using two-tailed Mann-Whitney test. F. CD95L immunohistochemical staining of breast tissues from patients with the indicated breast cancer subtype (20x magnification). Inset shows a 40× magnification of blood vessels. Stars indicate the tumor mass and black arrows show the blood vessels. G. Immunohistochemical staining analysis of consecutive sections of TNBC tissues stained with markers of blood (CD31+), or lymphatic (D2-40+) endothelium and CD95L. White arrowheads show a lymphatic vessel, black arrows depict a blood vessel.
Figure 2. Cl-CD95L induces cell migration through the activation of the p110β catalytic subunit of PI3K.

A. Cell migration of the indicated TNBC cells incubated with or without cl-CD95L (100 ng/mL) for 24 h was assessed using the Boyden chamber assay. Migrating cells were fixed and stained by Giemsa. For each experiment, five images of random fields were acquired. A representative image is shown. Bars=70 μm. B. **Left panels:** MDA-MB-231 cells were incubated with serum from healthy donors (control) or different TNBC women (P# for patient) for 24 h in Boyden chambers. Migrating cells were fixed and stained by Giemsa and a representative image is shown. Bars=70 μm. **Right panel:** Giemsa-stained migrating cells were lysed and absorbance was measured at a wavelength of 560 nm. Values represent the means ±SEM of three independent experiments. * p<0.05 as calculated using two-tailed Mann-Whitney test. C. MDA-MB-231 breast tumor cells infected with lentivirus encoding p110α- and p110β-targeting shRNA were stimulated in the presence (15 min) or absence (0 min) of cl-CD95L (100 ng/ml), and the levels of phosphorylated Akt and total Akt were analyzed by immunoblotting analysis. D. **Upper Panel:** The motility of the cells described in C was assessed using the Boyden chamber assay. **Lower Panel:** Migrating Giemsa-stained cells were lysed and absorbance was measured at a wavelength of 560 nm. Values represent the means ±SEM of three independently performed experiments. E. TNBC cells were incubated with 100 ng/mL of cl-CD95L for the indicated times and then lysed. CD95 was immunoprecipitated and the immune complex was resolved by SDS-PAGE and analyzed by western blot with the indicated antibodies. F. **Upper panel:** MDA-MB-468 cells transduced with lentivirus encoding the indicated shRNAs were incubated for 24 hours in the presence or absence (control) of cl-CD95L (100 ng/mL) and cell migration was assessed using Boyden chambers. **Lower panel:** Quantitative analyses of cell migration as described in B.
Values represent the means ±SEM of three independently performed experiments. * p<0.05 as calculated using two-tailed Mann-Whitney test.

**Figure 3.** cl-CD95L evokes an Orai1-driven Ca$^{2+}$ entry contributing to cell migration.

A. MDA-MB-468 cells were pre-incubated with 5 μM of BAPTA-AM or 500 ng/mL of BTP2 for 30 min and then treated or untreated (control) with 100 ng/mL cl-CD95L. Cells were then lysed and 100 μg of protein was loaded per lane. Immunoblot analysis was performed for phospho-ser473 Akt and total Akt. B. MDA-MB-468 cells were pre-incubated with 5 μM BAPTA-AM or 500 ng/mL BTP2 for 30 min and then treated or untreated (control) with 100 ng/mL cl-CD95L for 24 hours. Cell migration was assessed using the Boyden Chamber assay, and the amount of Giemsa-stained migrating cells was quantified by measuring absorbance (O.D. 560 nm). Values represent the means ± SD of three independent experiments. ** p<0.01 as calculated using a two-tailed Mann-Whitney test. C. MDA-MB-468 cells were infected with lentivirus encoding scrambled or Orai1-targeting shRNAs. The inset shows the immunoblot analysis of Orai1 silencing in transduced MDA-MB-468 cells. Tubulin was used as a loading control. Infected MDA-MB-468 cells were loaded with the Ca$^{2+}$ probe Fluo8. The cells were then stimulated with 100 ng/mL cl-CD95L (black arrow) and [Ca$^{2+}$]$_{i}$ was monitored via the ratio F/F$_{0}$ (relative Ca$^{2+}$[CYT]). Data represent mean ±SD of F/F$_{0}$ measured in n cells. D. The cells depicted in C were stimulated for the indicated times with 100 ng/mL of CD95L and the activation status of Akt (phosphorylation at serine 473) was evaluated by immunoblotting. E. MDA-MB-468 cells infected with lentivirus encoding scrambled or Orai1-targeting shRNAs were incubated for 24 hours in the presence or absence of cl-CD95L, and cell migration was assessed using Boyden Chamber assays. Migrating cells were stained (Giemsa) and lysed, and
the percentage of migrating cells was quantified by measuring absorbance (O.D. 560 nm). 

F. The cl-CD95-triggered Ca\textsuperscript{2+} response was monitored in MDA-MB-468 cells in which e-yes (left panel) or p110β (right panel)-expression was suppressed by RNA interference. Cells were pre-loaded with Fluo8 and then stimulated with 100 ng/mL of cl-CD95L, and [Ca\textsuperscript{2+}], was monitored. Data represent the mean ±SD of the Ca\textsuperscript{2+} response in n cells.

Figure 4. Nox-3-driven ROS production is instrumental in the CD95-mediated unconventional signaling pathway.

A. MDA-MB-231 cells were loaded with the ROS probe H2FDA and pre-incubated for 30 min with the NADPH oxidase inhibitors DPI (1 μM) or apocynin (100 μM) or DMSO (control). Cells were then stimulated for 30 min with 100 ng/mL of cl-CD95L. ROS production was analyzed by flow cytometry. 

B. MDA-MB-231 cells were pre-incubated for 30 min with DPI (1 μM) or apocynin (100 μM) and then stimulated in the presence or absence of cl-CD95L (100 ng/mL). C-yes was immunoprecipitated and its phosphorylation was assessed by immunoblotting.

C. MDA-MB-231 cells were incubated with 100 ng/mL of cl-CD95L for the indicated times and then lysed. CD95 was immunoprecipitated and the immune complex was resolved by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

D. MDA-MB-231 cells were infected with lentivirus encoding scrambled or three different p22\textsuperscript{phox} ShRNAs. These cells were treated with cl-CD95L (100 ng/mL) for indicated times, lysed, and 100 μg of protein per lane was resolved by 10% SDS-PAGE. The activation status of Akt and the src kinase family was monitored by immunoblotting for the detection of phosphorylation at Ser473 and at Tyr426, respectively. Total protein is used as loading control. Data are representative of three independent experiments.

E. Cell migration was assessed on the cells described in D in the
presence or absence of cl-CD95L (100 ng/mL) for 24 h using the Boyden chamber assay. Images are representative of three independent experiments. **F.** MDA-MB-231 cells were incubated with 100 ng/mL of cl-CD95L (MISC) or APO1-3 (DISC) for the indicated times and then lysed. CD95 was immunoprecipitated and the immune complex was resolved by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

**Figure 5. CD95-dependent EGFR activation implements the PI3K signaling pathway.**

**A.** MDA-MB-468 cells were stimulated for the indicated times with cl-CD95L (100 ng/ml) and cells were then lysed. Phosphorylation of EGFR at Tyr845 and of Akt at Ser473 was monitored. Total EGFR and Akt served as loading controls. **B.** MDA-MB-468 cells infected with lentivirus encoding c-yes shRNAs were stimulated for the indicated times with 100 ng/mL cl-CD95L and the cells were then lysed. The amounts of EGFR phosphorylation at Tyr845 and total EGFR were assessed by immunoblotting. **C.** MDA-MB-468 cells were stimulated for the indicated times with cl-CD95L (100 ng/mL), lysed and CD95 was immunoprecipitated. The immune complex was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. Total lysate was used as input. **D.** MDA-MB-468 cells were stimulated as in C. Cells were lysed and EGFR was immunoprecipitated. The immune complex was subjected to a SDS-PAGE and analyzed by immunoblotting. Total lysate was used as input. Data are representative of three independent experiments. **E.** MDA-MB-231 cells infected with lentivirus encoding two different EGFR-targeting shRNAs were incubated with (15) or without (0) cl-CD95L (100 ng/mL) for the indicated times and then lysed. Equal amounts of protein (100 µg) were separated by SDS-PAGE and Akt phosphorylation at Ser473 was monitored by immunoblotting. Total Akt served as a loading control. **F.** Cell migration was assessed in MDA-MB-231 cells described in E.
using the Boyden chamber assay. Data represent the means ± SD of three independent experiments.

Figure 6. CD95-mediated motility signaling pathway.
CD95L cell surface cleavage triggers a pro-metastatic signaling pathway in triple negative breast cancer.

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