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1 **Antibodies against the multi-faceted cathepsin D protein open new avenues for TNBC**
2 **immunotherapy**

3

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18

19 **Running Title:** Antibody-based immunomodulatory therapy of triple-negative breast cancer

20

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22 paclitaxel, enzalutamide

23

24

25

ABSTRACT

Triple-negative breast cancer (TNBC) is a heterogeneous breast cancer subtype characterized by aggressive clinical behavior and poor prognosis. The immune landscape associated with TNBC often reveals high immunogenicity. Therefore, immunotherapy, which has demonstrated its efficacy in different cancer types, could be a promising strategy for TNBC, given the limited therapeutic options currently available besides conventional chemotherapy. The aspartic protease cathepsin D (cath-D) is a tumor cell-associated extracellular protein with pro-tumor activity, a marker of poor prognosis, and a target for antibody-based therapy in TNBC. This commentary provides a synopsis/narrative summary of the development of anti-cath-D antibodies in different formats, their key roles in restoring the anti-tumor immunity, particularly via activation of tumor-infiltrating natural killer cells, and their dual anti-tumor effects on cancer cells and stromal cancer-associated fibroblasts, suggesting their interest for clinical use in the light of the current clinical knowledge on TNBC.

Introduction

The aspartic protease cathepsin D (cath-D), a marker of poor prognosis in breast cancer (BC)^{1 2}, is overproduced by BC cells and hypersecreted in the tumor microenvironment³. Overexpressed cath-D exhibits tumor-promoting activity in BC by modulating cancer and stromal cell proliferation, tumor growth and angiogenesis, tumor cell apoptosis, and metastasis formation⁴⁻⁸. Cath-D was proposed as a potential therapeutic target to enhance anticancer drug-induced apoptosis⁹. In BC, extracellular cath-D displays pro-tumor roles through proteolytic cleavage of stromal/tumor components and also through non-proteolytic mechanisms by binding to receptors expressed on the surface of tumor cells and stromal cells, such as cancer-associated fibroblasts (CAFs). Secreted cath-D can alter the local extracellular matrix by cleaving collagens, fibronectin, proteoglycans, matricellular proteins (*e.g.*, SPARC)¹⁰, and by releasing growth factors trapped in the extracellular matrix (*e.g.*, FGF2)¹¹. Secreted cath-D can also disrupt the anti-tumor immune landscape by cleaving chemokines/cytokines involved in immune cell recruitment and activation^{12 13}. In the tumor microenvironment, cath-D can trigger the proteolytic cascade by activating the cathepsin B and L precursors¹⁴, or by degrading protease inhibitors (*e.g.*, cystatin C)¹⁵. Concerning its non-proteolytic mechanisms of action, cath-D promotes BC cell

1 proliferation *via* its pro-peptide ⁵, triggers breast fibroblast growth by binding to the LRP1 receptor¹⁶,
2 and induces endothelial cell proliferation and migration *via* the ERK and AKT signaling pathways¹⁷.
3 Therefore, extracellular cath-D could be a novel molecular target in BC for antibody-based therapy to
4 trap the abnormal pool of the secreted protease. In this commentary, we show that anti-cath-D antibody
5 therapy can inhibit tumor growth, restore the anti-tumor immunity, particularly by activating tumor-
6 infiltrating natural killer (NK) cells that target both cancer and stromal cells (CAFs), and improve the
7 therapeutic index of chemotherapy and hormone therapy. We also discuss what needs to be investigated
8 to complete the preclinical characterization of these antibodies in view of their clinical development.

9

10 **The fully-human anti-cath-D antibody F1 as a new immunomodulator in triple-negative BC** 11 **(TNBC)**

12 The first-in-class F1, a fully human anti-cath-D IgG1 originally isolated by phage display, localizes at
13 the tumor site and inhibits the growth of MDA-MB-231 (TNBC-derived) cell xenografts and patient-
14 derived xenografts (Figure 1A). Mechanistically, F1 activates NK cells and prevents the recruitment of
15 M2-polarized tumor-associated macrophages (TAM) and myeloid-derived immunosuppressive
16 populations (MDSCs) in TNBC cell xenografts in nude mice (Figure 1A)¹⁸. Interestingly, the Fc-
17 aglycosylated F1 variant, where the N297A mutation prevents binding to the Fc γ receptors, no longer
18 induces NK cell activation, and is associated with reduced tumor growth inhibition¹⁸. This demonstrates
19 the essential role of the Fc part of the F1 antibody for cath-D-specific targeted therapy, possibly via NK
20 cell activation and NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC). It also suggests
21 that cath-D is a prime target for monoclonal antibody-based therapies with immunomodulatory
22 activity¹⁸.

23

24 **F1M1 immunomodulatory activity in an immunocompetent mouse model of TNBC**

25 To explore the overall impact of anti-cath-D antibodies on the immune landscape of TNBC, we next
26 investigated the therapeutic effect and immunomodulatory activity of the anti-cath-D murine IgG2a
27 antibody F1 and of its improved version F1M1 with aglycosylated Fab in an immunocompetent mouse
28 model of TNBC (C57BL/6 mice harboring E0771 cell grafts)¹⁹. This was possible because F1 and F1M1

1 cross-react with mouse cath-D. This study showed that both F1 and F1M1 inhibit tumor growth in the
2 highly-immunogenic E0771 mouse model. Both antibodies promoted the innate antitumor immunity by
3 preventing the recruitment of immunosuppressive M2-TAMs and by activating NK cells in the tumor
4 microenvironment (Figure 1B). This translated into a reduction of PD-L1⁺ tolerogenic T cells and of
5 exhaustion markers, including LAG3 and TIGIT, in the tumor microenvironment, possibly locally
6 supported by enhanced activation of antigen-presenting cell (M1-polarized CD86⁺ TAMs and CD86⁺
7 conventional type 1 dendritic, cDC1, cells) functions and upregulation of MHC class 1 molecules, β 2-
8 microglobulin and Fc γ Rs (Figure 1B). Thus, F1 and F1M1 remodel the tumor immune landscape by
9 triggering both innate and adaptive anti-tumor immunity in an immunocompetent mouse model of
10 TNBC¹⁹. Although few data are available on cath-D roles in anti-tumor immunity, it has been shown
11 that cath-D promotes M2-TAM polarization through TGFBI-CCL20 signaling in TNBC *in vivo*²⁰. To
12 further validate F1M1 efficacy in preclinical settings, it would be important to include other
13 immunogenic TNBC mouse models. Unfortunately, the 4T1 TNBC mouse cell line does not secrete the
14 cath-D precursor¹⁹. If EMT-6 cells secrete the cath-D precursor, the EMT-6 TNBC model in Balb/c mice
15 could be an interesting alternative. This model has already been used to study the efficacy of anti-
16 immune checkpoint antibodies (against PD1, PDL1, CTLA4) and chemotherapy (carboplatin) and
17 radiotherapy, either alone or in combination²¹.

18

19 **Fc-engineering of F1M1 for future clinical development in TNBC**

20 Based on our previous studies suggesting that F1 and F1M1 can activate tumor-infiltrating NK cells^{18,19},
21 we protein-engineered the Fc part of the F1M1 IgG1 to enhance NK cell activation and potentiate its
22 anti-tumor efficacy in TNBC, as previously demonstrated^{22,23}. As Fc dependence is often a prerequisite
23 to ensure antibody efficacy in clinical settings, deciphering NK cell activation after F1M1 administration
24 was an essential step for its future clinical development. The Fc-optimized F1M1-Fc⁺ antibody (derived
25 from F1M1 by introducing the S239D, H268F, S324T, and I332E mutations) displays enhanced affinity
26 for CD16a/Fc γ RIIIa regardless of 158F/V allotypes. *In vitro*, among the different anti-cath-D F1-derived
27 antibodies, F1M1-Fc⁺ most potently activated NK cells, with increased CD107a cell surface expression

1 and intracellular IFN γ production (Figure 1C), and also triggered ADCC of TNBC cells and stromal
2 CAFs. This dual cytotoxic effect in cancer and stromal cells following cath-D targeting was never
3 described before (Figure 1C). F1M1-Fc⁺ showed improved antitumor potency compared to F1M1, and
4 triggered NK cell recruitment, activation and cytotoxic activity in MDA-MB-231 cell xenografts in nude
5 mice (Figure 1C)²³. Following F1M1-Fc⁺ treatment, the expression of CD107a (at the cell surface) and
6 intracellular granzyme B was significantly increased in NK cells that infiltrated MDA-MB-231 cell
7 xenografts (Figure 1C). Remarkably, F1M1-Fc⁺ induced the tumor recruitment of immature CD27⁻
8 CD11b⁻ and mature stage 1 CD27⁺CD11b⁻ NK cells undergoing activation, as indicated by the
9 concomitant *Eomes* upregulation (Figure 1C). Moreover, NK cell depletion, by treatment with the anti-
10 asialo GM1 antibody, impaired F1M1-Fc⁺ effect in nude mice harboring MDA-MB-231 cell xenografts,
11 demonstrating the key role of tumor-infiltrating NK cells in F1M1-Fc⁺ antitumor activity. Recent studies
12 and clinical trials underlined that NK cell-based immunotherapy can awake the innate anticancer
13 response, particularly against tumor metastases²⁴. One hypothesis is that F1M1-Fc⁺ targets NK cells to
14 primary tumors and also to metastases, sustaining dormancy as demonstrated in TNBC²⁵. Importantly,
15 F1M1-Fc⁺ ability to trigger ADCC in CAFs highlights its dual action in tumor and stromal cells to
16 modulate the whole tumor architecture.

17

18 **Combinatorial strategies with optimized anti-cath-D antibodies to tackle the TNBC** 19 **microenvironment**

20 In the clinic, antibody-based therapies are usually combined with chemotherapy, hormone-therapy
21 and/or other targeted therapies for TNBC management. In metastatic TNBC, the immune checkpoint
22 inhibitor (ICI) pembrolizumab (anti-PD-L1 antibody) in association with chemotherapy shows
23 improved overall and progression-free survival compared with chemotherapy alone²⁶. Moreover, the
24 androgen receptor (AR) inhibitor enzalutamide shows clinical activity and is well tolerated in patients
25 with early and advanced AR⁺ TNBC²⁷. Therefore, we assessed the added value of the F1M1-Fc⁺ lead
26 antibody in combination with chemotherapy and hormonotherapy. F1M1-Fc⁺ improved paclitaxel and
27 enzalutamide efficacy²³, suggesting its clinical relevance in combination therapies (Figure 1C).
28 Comparison of the therapeutic efficacy of the anti-cath-D antibodies in the highly-immunogenic TNBC

1 E0771 and the immune-excluded BC TUBO cell graft models suggests that their antitumor activity is
2 immune cell-mediated¹⁹. Therefore, they could be efficient in combination with ICI in ‘hot’ tumors¹⁹.
3 The highly-immunogenic E0771 cell-based TNBC mouse model could be a tool of choice to assess the
4 combination of F1M1 with anti-PD1 and anti-PD-L1 ICIs because E0771 cells express these checkpoint
5 markers²⁸.

6

7 **Conclusions and future challenges**

8 Classically, antibody-based therapy targets a receptor overexpressed by cancer cells (e.g., EGFR by
9 cetuximab) or by stromal cells (e.g., FAP by sibrotuzumab). Overall, in preclinical models of TNBC,
10 anti-cath-D antibody-based therapy inhibits tumor growth, triggers the anti-tumor innate and adaptive
11 immunity, and has a dual role on both cancer cells and CAFs through ADCC mechanisms, suggesting
12 diverse effects on the whole tumor architecture^{18 19 23}. This could open new therapeutic avenues for
13 designing smarter antibodies that target a soluble pro-tumoral antigen specifically hypersecreted within
14 the tumor and capable of interacting with cancer cells and also tumor stroma cells.

15 In parallel to the development of anti-cath-D antibodies, we analyzed cath-D expression in TNBC tissue
16 microarrays to identify which patients could benefit from this novel targeted therapy in order to develop
17 personalized medicine. AR⁺/cath-D⁺ co-expression identifies a subgroup of TNBC with poor prognosis
18 that could potentially be eligible for combination therapy with an anti-cath-D antibody²⁹. Importantly, a
19 new classification of TNBC using immunohistochemistry has been developed and can be easily used by
20 hospitals, compared with genetic and genomic analyses that are difficult to use in routine practice, to
21 stratify patients for selecting the best treatment combinations³⁰. As anti-cath-D antibodies can improve
22 chemotherapy and hormone therapy in TNBC²³ and have immunomodulatory activity, it could be
23 interesting to test them in combination with ICIs.

24 Regarding the future positioning of anti-cath-D antibody-based therapy, naked antibodies could be used
25 to overcome (or reduce) ICI-induced toxicity, as a second line treatment after anti-PD-1/anti-PD-L1
26 antibodies in patients with metastatic TNBC, or in combination to synergize ICI effects for enhancing
27 the patients’ immunological anti-tumor defenses. They could also be used in patients with PD-L1-
28 negative (<1% PD-L1⁺ immune cells) metastatic TNBC for whom ICI-based immunotherapy is not

1 indicated. Anti-cath-D antibodies could be used in combination with antiandrogens in patients with
2 hormone-dependent (AR⁺/cath-D⁺) metastatic TNBC, or as a second-line treatment in (AR⁺/cath-D⁺)
3 metastatic TNBC that has become resistant to hormone therapy.
4 Cath-D is upregulated, and abnormally secreted in the tumor microenvironment of many cancer types,
5 including ovarian, pancreatic, melanoma, squamous cell carcinoma, hepatocarcinoma, prostate,
6 glioblastoma and colorectal cancer, and displays tumor-promoting roles. Therefore, anti-cath-D therapy
7 could be tested also in other tumor types, particularly the most aggressive cancers for which there is no
8 effective targeted therapy and/or with high infiltrating stroma, particularly pancreatic cancer and
9 prostate cancer. This strategy of using antibodies to target protumor proteins that are specifically
10 hypersecreted in the tumor microenvironment and modulate tumor architecture, and therefore with a
11 triple impact on cancer, stromal, and immune cells, could be relevant also for other hypersecreted
12 proteins.

13

14 **Consent for publication**

15 “Not applicable”

16

17 **Availability of data and material**

18 “Not applicable”

19 **Competing interests**

20 The authors declare no potential conflicts of interest.

21

22 **Ethics and study approvals**

23 Mouse experiments were performed in compliance with the French regulations and ethical guidelines
24 for experimental animal studies in an accredited establishment (Agreement No. #31135
25 2021042212479661). The study approval for PDXs was previously published. For
26 immunohistochemistry (IHC), TNBC biopsy samples were provided by the biological resource center
27 (Biobank number BB-0033-00059) after approval by the Montpellier Cancer Institute Institutional
28 Review Board, following the French national Ethics and Legal dispositions for patients’ information

1 and consent. For TNBC cytosols, patient samples were processed according to the French Public Health
2 Code (law n°2004-800, articles L. 1243-4 and R. 1243-61), and the biological resources center has been
3 authorized (authorization number: AC-2008-700; Val d'Aurelle, ICM, Montpellier) to deliver human
4 samples for scientific research. All patients were informed before surgery that their surgical specimens
5 might be used for research purposes. For human primary NK cell isolation and expansion, this work
6 benefited from umbilical cord blood units and John De Vos' expertise (head of the Biological Resource
7 Center Collection of the University Hospital of Montpellier, [http://www.chumontpellier.](http://www.chumontpellier.fr/en/platforms)
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10

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18

19

AUTHOR CONTRIBUTIONS

20 PDR, TD, AM, TC and ELC prepared the manuscript. PDR, TD, AM, VLM, PR, SG, TC and ELC
21 proof-read and finalized the manuscript. PDR and TD contributed equally to this work. ELC acted as
22 guarantor.

23

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- 10

Legend to Figure

Figure 1. Antibody targeting the multi-faceted cathepsin D protein as a novel clinical opportunity for TNBC immunotherapy.

(A) Immunotherapy of TNBC with the F1 anti-cath-D targeting antibody¹⁸. F1 anti-tumor response is triggered through NK cell activation via *IL15* upregulation, associated with granzyme B and perforin production, and IFN γ release. F1 also prevents the recruitment of M2-TAMs and MDSCs, a specific effect associated with a less immunosuppressive tumor microenvironment highlighted by TGF- β decrease.

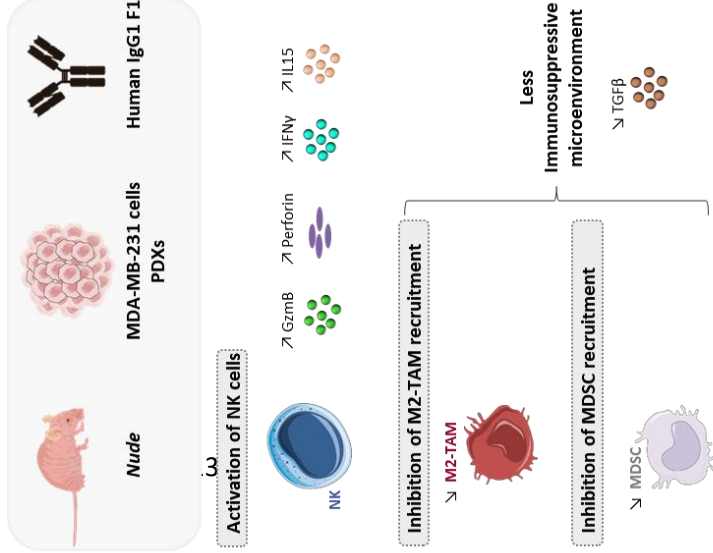
(B) Anti-cath-D immunotherapy with F1 and F1M1 triggers both innate and adaptive antitumor immunity in an immunocompetent mouse model of TNBC¹⁹. The F1 and F1M1 antibodies in the mouse IgG2a format promote the innate antitumor immunity by activating NK cells and preventing the recruitment of immunosuppressive M2-TAMs in the tumor microenvironment of C57BL/6 mice harboring E0771 cell grafts. They also enhance activation of anti-tumor antigen-presenting cells (CD86⁺ M1-polarized TAMs and cDC1 cells), supporting the reduction of PD-L1⁺ tolerogenic CD4⁺ and CD8⁺ T cells and exhaustion markers, including LAG3 and TIGIT, in the tumor microenvironment.

(C) Generation of the Fc-engineered F1M1-Fc⁺ anti-cath-D antibody as a novel therapeutic opportunity for TNBC²³. F1M1-Fc⁺ enhances *in vitro* NK cell activation and ADCC of both TNBC cells and CAFs. F1M1-Fc⁺ triggers tumor-infiltrating NK cell recruitment and cytotoxic activity in TNBC. In combination therapies, F1M1-Fc⁺ improves the therapeutic efficacy of paclitaxel and enzalutamide in TNBC.

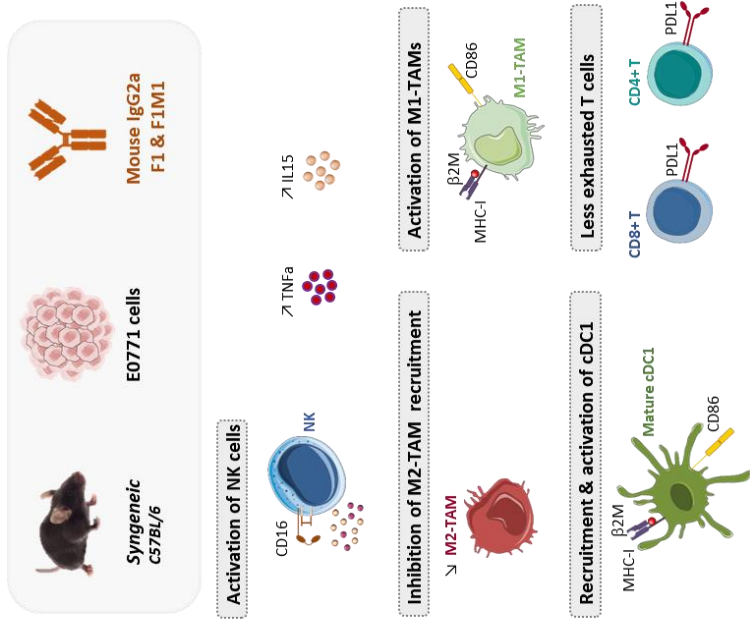
Immunotherapy of TNBC with cathepsin D-targeting antibodies



A F1-mediated antitumor response



B F1 & F1M1-mediated innate and adaptive immunity



C F1M1-Fc⁺-enhanced antitumor response

