ERRα Expression in Bone Metastases Leads to an Exacerbated Antitumor Immune Response

Mathilde Bouchet1,2, Alexandra Lainé2,3, Cyril Boyault4, Mathilde Propponet-Guerault5, Emmanuelle Meugnier6, Lamia Bouazza1,2, Casina W.S. Kan1,2, Sandra Geraci1,2, Soumaya El-Moghrabi1,2, Hector Hernandez-Vargas2, Claire Benetollo1,2,6, Yuji Yoshiko7, Martine Duterque-Coquillaud8, Philippe Clézardin1,2, Julien C. Marie2,3, and Edith Bonnelye1,2

ABSTRACT

Bone is the most common metastatic site for breast cancer. Although the estrogen-related receptor alpha (ERRα) has been implicated in breast cancer cell dissemination to the bone from the primary tumor, its role after tumor cell anchorage in the bone microenvironment remains elusive. Here, we reveal that ERRα inhibits the progression of breast metastases of breast cancer cells by increasing the immune activity of the bone microenvironment. Overexpression of ERRα in breast cancer bone metastases induced expression of chemokines CCL17 and CCL20 and repressed production of TGFβ. Subsequently, CD8+ T lymphocytes recruited to bone metastases escaped TGFβ signaling control and were endowed with exacerbated cytotoxic features, resulting in significant reduction in metastases. The clinical relevance of our findings in mice was confirmed in over 240 patients with breast cancer. Thus, this study reveals that ERRα regulates immune properties in the bone microenvironment that contributes to decreasing metastatic growth.

Significance: This study places ERRα at the interplay between the immune response and bone metastases of breast cancer, highlighting a potential target for intervention in advanced disease.

Introduction

Bone metastases are a frequent complication of cancer, occurring in up to 70% of patients with advanced breast cancer, and are associated with both high morbidity and elevated mortality (1–3). The progression of bone metastases relies on the ability of the malignant cell colonizing the bone and to modify bone microenvironment allowing the release of bone-stored factors including TGFβ, bone morphogenetic protein, or insulin growth factor, which in turn stimulate bone metastasis progression (2, 4). However, treatments that mainly involved antiresorptive agents of the bone aimed to suppress osteoclast activity (5), implying that other mechanisms than the activation of osteoclasts by tumor cells are involved in modulating bone metastasis growth. The immune cells present in the bone and particularly activated CD8+ T lymphocytes can repress the progression of breast cancer osteolytic bone metastasis (5–8). However, whether bone metastasis can influence the activation of immune cells present in the bone and by which mechanisms is totally unknown.

The estrogen-related receptor alpha (ERRα, or NR3B1 according to the Nuclear Receptors Nomenclature Committee, 1999) is overexpressed in 55% of breast tumors (9, 10). Though ERRα shares structural similarities with the estrogen receptors α/β, it does not bind estrogens and no natural ligand has yet been found (11), though several molecules can either increase or decrease ERRα activity, such as the inverse agonists XCT790 or C29 (12, 13). ERRα is mainly involved in the adaptive bioenergetics response (11). In cancer, beside angiogenesis, ERRα is strongly linked to tumor cell invasion (14, 15). Notably, ERRα-positive tumors are associated with more invasive breast cancer and a higher risk of recurrence (9, 14). The overexpression of ERRα in breast cancer promotes tumor growth in the mammary gland and breast cancer metastatic dissemination to the bone (16). However, the role of ERRαs in bone metastasis outcome once they have anchorage in the bone microenvironment remains elusive.

In this study, using loss and gain of expression of ERRα, as well as chemical inhibitors, we demonstrated that ERRα enhanced the ability of breast cancer cell established in the bone to recruit activated CD8+ T cells to the bone. In addition, ERRα expression on breast cancer cells repressed their ability to produce TGFβ, a potent immune-suppressive cytokine. Subsequently, TGFβ signaling was impaired in T cells infiltrating the bone, and CD8+ T-cell cytotoxic function exacerbated, leading to metastatic progression. Altogether, our work assigns a totally unexpected role to ERRα, revealing that the expression of this orphan receptor on the breast cancer metastases promotes an efficient antitumor immune response once the tumor cells are settled in bone.
Materials and Methods

Cell lines

The mouse triple-negative breast cancer (TNBC) cell line 4T1 (year 2012; ATCC: lot 58603185-CRL-2539) and human luminal MCF7 (year 2012; ATCC-HTB-22 Lot: 86012803) were obtained from the ATCC. MDA-MB-231/BO2-FRT (BO2) breast cancer cells, a subpopulation of the human MDA-MB-231 breast cancer line (TNBC), were selected for their high efficiency to metastasize to bone (17). These cell lines were tested for authentication by DNA fingerprinting using the short tandem repeat method in 2014. TNBC cell lines and MCF7 were cultured in DMEM or RPMI-1640 (Life Technologies) medium, respectively, supplemented with 10% FBS (Perbio) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO2 incubator. Cells lines were tested for Mycoplasma regularly. Mouse and human ESRRA (ERRx) cDNA and the dominant-negative coactivator domain AF2 (AF2) mutants were described previously (16, 18). Briefly, pSR-ERRxWT and pEcmv-ERRxAF2 or respective empty vectors (CT) constructs were transfected into parental 4T1 cells and cultured for 4 weeks in puromycin (2 μg/mL; Life Technologies). Three independent clones were obtained from pSR-ERRxWT transfection (4T1-ERRx) and from pEcmv-ERRxAF2 transfection (4T1-ERRxAF2). Two independent clones were obtained from empty vector transfection respectively, pSR-CTR (4T1-CT) and pEcmv-4T1-CT (4T1-CTα2). For MCF7 clones, a mix containing 1.5 μg Retroviral pLPCX-Human-ERRxWT, pLPCX-HumanERRxAF2, or empty vector and 0.5 μg pCMV-VSV-G envelope vector (Cell Biolabs) was used previously (16). 4T1-ERRx and 4T1-ERRxAF2 (pool of 3 clones each) cells were treated for 24 hours with the ERRx inverse-agonists XCT790 (Sigma) or C29 (AGV Discovery) at 1 and 5 μM/l, respectively, as described (12, 13, 16). DMSO was used as a vehicle (Veh).

Animal studies

Six-week-old BALB/c female mice were purchased from Janvier and housed in a specific pathogen-free facility (ALECS platform; Faculté de Médecine Laennec, Lyon, France). Bone metastasis experiments were performed by inoculating intra-arterially either 4T1-CT (pool of 2 clones) in parallel with 4T1-ERRx (pool of 3 clones) or 4T1-CTα2 (pool of 2 clones) in parallel with 4T1-ERRxAF2 (pool of 3 clones) cells (5 × 10^5 cells in 100 μL of PBS). Radiographs (LifeRay HM Plus, Ferrania) of animals were taken 5 days after inoculation using X-ray (MX-20; Faxitron X-ray Corporation). The extent of bone destruction for each animal was expressed in mm^2. Animals were sacrificed and hind limbs were then collected for histology and histomorphometric analysis. Tibiae were scanned using microcomputed tomography (Skyscan1076, Skyscan) with an 8.8 voxel size and an X-ray tube (50 kV; 80 mA) with 0.5 mm tomography (Skyscan1076, Skyscan) with an 8.8 voxel size and an X-ray tube (50 kV; 80 mA) with 0.5 mm tomography (Skyscan1076, Skyscan). Briefer, pSR-ERRxWT and pEcmv-ERRxAF2 or respective empty vectors (CT) constructs were transfected into parental 4T1 cells and cultured for 4 weeks in puromycin (2 μg/mL; Life Technologies). Three independent clones were obtained from pSR-ERRxWT transfection (4T1-ERRx) and from pEcmv-ERRxAF2 transfection (4T1-ERRxAF2). Two independent clones were obtained from empty vector transfection respectively, pSR-CTR (4T1-CT) and pEcmv-4T1-CT (4T1-CTα2). For MCF7 clones, a mix containing 1.5 μg Retroviral pLPCX-Human-ERRxWT, pLPCX-HumanERRxAF2, or empty vector and 0.5 μg pCMV-VSV-G envelope vector (Cell Biolabs) was used previously (16). 4T1-ERRx and 4T1-ERRxAF2 (pool of 3 clones each) cells were treated for 24 hours with the ERRx inverse-agonists XCT790 (Sigma) or C29 (AGV Discovery) at 1 and 5 μM/l, respectively, as described (12, 13, 16). DMSO was used as a vehicle (Veh).

Ethics statement

Mice were handled according to the French Ministerial Decree No. 87–848 of October 19, 1987. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the Université-Lyon1 (France; ethic committee CEEA-55 Comité d’Ethique en Expérimentation Animale-DR2014-44-DR2015-28).

Human sample meta-genomic analysis

Correlation analyses were performed using published datasets downloaded from the Gene Expression Omnibus including primary tumor, no metastases, visceral + bone, or only bone metastasis (GSE12276-GSE2034-GSE2603; n = 248; refs. 19–21). Z-scores were calculated on normalized data of each dataset by subtracting the population mean from individual expression values for each gene and then dividing the difference by the population SD.

Sign arrays

The expression levels of several chemokines known to influence T-cell chemotaxis were obtained by qPCR Sign Arrays (Cytokines Array and Inflammation Array). Indeed, two qPCR Sign Arrays, Cytokines and Inflammation Arrays (AnyGenes, CT1M1-IFM1, ClinSciences), were used to quantify expression of cytokines, chemokines, and growth factors. Total RNA was extracted from 4T1-CT and 4T1-ERRx cells, and 2 μg were reverse-transcribed as previously described (16). Real-time PCR was performed according to the manufacturer’s instructions. Two heat maps were generated using the heatmap.2 function in the gplots library of R (version 3.5.1). Only regulations that were reproducible between the two arrays are presented.

Protein–protein interaction network reconstruction and analysis

The protein–protein interaction network with BIOGRID (release 3.4.160) from Homo sapiens with PSICQUIC (Proteomics-Standard-Initiative-Common-QUery-InterfaCe) retrieved (10242018) and Cytoscape environment was used (16). A BIOGRID (https://thebiogrid.org/)-based custom approach was used to define a protein interactome of the following proteins: ESRRA-CCL17-CCL20-OPG-NRP1-SCRC1-SRC2-SCRC3-PGC1A-PGC1B-CCR4 and CCR6. The resulting interactome encompasses 911 proteins (hereby defined as “Extended Network of ESRRA, CCL17, CCL20”, reachable on Nedx webserver, 10.18119/N9RK5C: containing 101 proteins) acknowledging connections that may support ESRRA signaling (Supplementary Fig. S2; ref. 16). We overlaid and extracted information from the Gene Ontology consortium to pinpoint proteins that are already known to be involved in the immune system process, as well as T- and B-cell homeostasis (GO-IDs: 0002376, 0043029, 0001782) to create “Minimal Network specific to immune response to tumor” (containing 52 proteins). To determine the connectors between CCL17, CCL20, and ESRRA, a custom approach combining shortest path and connectivity degree analysis was applied to determine a “Minimal Network of ESRRA, CCL17, CCL20” (reachable on Nedx webserver, 10.18119/N9RK5C; containing 101 proteins) acknowledging connections that may support ESRRA signaling (Supplementary Fig. S2; ref. 16). We overlaid and extracted information from the Gene Ontology consortium to pinpoint proteins that are already known to be involved in the immune system process, as well as T- and B-cell homeostasis (GO-IDs: 0002376, 0043029, 0001782) to create “Minimal Network specific to immune response to tumor” (containing 52 proteins). To determine the connectors between ESRRA and CCL17, ESRRA and CCL20, a shortest path was applied to the “Minimal Network of ESRRA, CCL17 and CCL20” (16, 22, 23).

Ex vivo cell preparation

For hind limbs, muscles were removed, and bones were sliced and then incubated at 37°C with a 1/10 solution of collagenase hyaluronidase (Stem Cell) for 2 hours. Bones were then mechanically disrupted...
with a syringe plunge, filtered, and cells were collected. For lung metastases (LM), lungs were crushed with a syringe plunge on a filter (100 µm; BD Bioscience) and cells were collected. Cells released from lungs and bones were incubated at 37°C in the presence of DMEM (Life Technologies) supplemented with 10% (v/v) FBS (Perbio/Thermo Scientific) and 6-thioguanine (Sigma A8882; 10 µg/mL) for 2 weeks. The cells were then counted after being stained using Crystal Violet (RAL diagnostic 317980).

**Flow cytometry**

Cells from spleen and lungs, obtained after mechanical disruption, and flushed bone marrow cells were preincubated with anti-CD16/32 (93 clone, Biolegend) and stained for surface marker for 30 minutes at 4°C with the following antibodies: anti-CD45 (30-F11 clone, BD or ebiosciences), anti-CD3e (145-2C11 clone, BD), anti-CD4 (GK1.5 clone, BD), anti-CD8 (53-6.7 clone, BD or ebiosciences), anti-CD19 (1D3 clone, BD), anti-CD11b (M1/70 clone, ebiosciences), anti-CD11c (N418 clone, ebiosciences), anti-CCR4 (2G12 clone Biolegend), anti-CCR6 (29-2217 clone Biolegend), anti-Ly6C (AL21 clone, BD), anti-Ly6G (1A8 clone, BD), anti-F4/80 (BM8 clone, Biolegend), anti-CD107a (LAMP-1; 1D4B clone, BD), and anti-FasL (MFL3 clone, eBiosciences). For cytokine production, cells were first incubated for 4 hours with PMA (P1585-1MG, Sigma), ionomycin (1000 µmol/L, Sigma), and 6-thioguanine (Sigma A8882; 10 µg/mL) for 2 weeks. The cells were then counted after being stained using Crystal Violet (RAL diagnostic 317980).

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay**

Bone sections were deparaffinized and rehydrated followed by permeabilization with 0.2% Triton (T9284, Sigma) and digestion with proteinase K (1 µg/mL; K182001; Thermo Fisher). For positive control, sections were incubated with DNase I at 1 mg/mL (Sigma, 11284932001). Sections were then incubated with biotin-16-dUTP (Sigma, 11093070910) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) enzyme (Sigma, 11767305001) in deoxynucleotidyltransferase buffer (Tris-HCl 125 mmol/L, Euromedex, EU0011), sodium cacodylate 200 mmol/L (Sigma, C0250), BSA 6 mmol/L (Sigma, A7906), and CoCl2 1 mmol/L (Sigma, 15862-1ml-F) at 37°C for 60 minutes in a humid atmosphere. Sections were washed in stop buffer [300 mmol/L NaCl (Sigma, S3014) and 30 mmol/L Na2HPO4·12H2O·5 sodium citrate (Sigma, 71406)] and blocked with 2% BSA (Sigma, A7906). Sections were then labeled with streptavidin–phycoerythrin (eBiosciences, 12-4317-87) and DAPI (Euromedex, 1050-A) and mounted with Fluoromount [Sigma, F4680-25 mL; upright microscope zeiss axioimager (sip 60549)].

**Real-time RT-PCR**

Total RNAs from three independent batches of each clone of 4T1, MCF7 and 80B2 cell (CT, ERRα, and ERRαRtAF2) were extracted with Trizol reagent (Life Technologies), and 2 µg were reverse-transcribed using qScript cDNA SuperMix (Quanta-Biosciences). Real-time PCR was performed on a Mastercycler-ep-Realplex (Eppendorf) with primers specific to human and mouse genes (Supplementary Table S1) using Quantifast SYBR-Green (Life Technologies) according to the manufacturer’s instructions. The ribosomal protein RPL32 (L32) gene was used as a housekeeping gene for quantification, and relative results expressed as fold differences equal to 2^ΔΔCt.

**Statistical analyses**

Data were analyzed statistically using either the nonparametric Mann–Whitney U test or unpaired t test for in vivo studies [n = 10 mice for each group (bioStatTVG), unblinded studies]. In vitro data on bone were confirmed (n = 3) on smaller groups (n = 4). In vitro assays were repeated at least twice and performed on triplicate samples. Data were analyzed using ANOVA and paired Student t test to assess the differences between groups. All data are presented as mean ± SEM with similar variances between groups. Correlation scores for meta-analysis were calculated using the Pearson correlation coefficient. Statistical significance was determined by GraphPad Prism v5.02 using the two-sided Student t test. All statistical analyses were performed using the GraphPad Prism software. P values less than 0.05 were considered statistically significant.
Results

ERRα expression in breast cancer cells inhibits metastases growth in bones

In order to assess the role of ERα in breast cancer cells after tumor cell anchorage in the bone microenvironment, BALB/c mice were intra-arterially injected with first a pool of three independent 4T1 tumor cell clones overexpressing ERα (4T1-ERRα) and a pool of two 4T1 tumor cell clones transfected with empty vector controls (4T1-CT; ref. 16). Remarkably, 15 days later, radiographic analysis revealed that animals bearing 4T1-ERRα tumors had osteolytic lesions that were 70% smaller than those of mice bearing 4T1-CT tumors (9.39 ± 2.6 vs. 3.08 ± 1.45 mm²; Fig. 1A and B). The inhibitory effect of ERα on breast cancer cell growth was associated with mild bone destruction (Fig. 1C–E). Histologic and histomorphometric analyses also demonstrated the limitation of bone metastasis progression when breast cancer cells overexpressed ERα (Fig. 1F and G). In clear contrast, when clones expressing a dominant-negative form (4T1-ERRαAf2) with their respective controls clones (4T1-CTa2) were injected, we found a 60% increase in osteolytic lesions in animals bearing ERαAf2 tumors compared with control (4T1-CTa2) mice (3.82 ± 1.99 vs. 9.27 ± 2.064 mm²), leading to their earlier sacrifice, prior 4T1-CTa2(Af2) bone metastasis reached the percentage of osteolysis observed in 4T1-CT (Fig. 1H and I). Concomitantly, increased bone destruction and tumor burden were observed (Fig. 1J–N). Given that 4T1 cells also colonize the lung (16), we analyzed the effects of ERα expression on the development of LM. As opposed to the bone, both numbers of LM and numbers of breast cancer colonies extracted from the lung were independent of the expression levels of ERα in 4T1 cells (Fig. 2A–F). Of note, the ERα overexpression in LM was observed in animals bearing 4T1-ERRα tumors compared with control groups (Fig. 2G). Taken together, this first set of data reveals that the overexpression of ERα in breast cancer cells prevents their growth in the bone and suggests that ERα expression in breast cancer may affect the bone microenvironment to prevent bone metastasis progression.

ERRα expression in breast cancer cells increases T-cell antitumor response in the bone

Given the importance of the immune response in the control of tumor growth, in particular metastases (5–8), we next analyzed the effects of the expression of ERα by breast cancer cells on the bone immune system. It is worth noting that no significant effect was observed on innate cells including dendritic cells, macrophages, with the exception of slight decrease in neutrophils (15%) in the bone colonized by 4T1-ERRα compared with 4T1-CT cells (Supplementary Fig. S3A–S3C). However, we found that metastatic legs of animals bearing 4T1-ERRα tumors contained 5 more T cells than those colonized with 4T1-CT cells (Fig. 3A). Moreover, in line with the larger 4T1-CT bone metastasis observed (Fig. 1), which developed at the expense of the bone marrow that is largely depicted to sustain all larger 4T1-CT bone metastasis observed (Fig. 3C–E). In clear contrast, the depletion of CD8⁺ T cells was sufficient (Fig. 4A) to increase 4T1-ERRα bone metastasis progression (Fig. 4B–E). This set of data suggests that the expression of ERα in breast cancer cells influences the CD8⁺ T-cell homeostasis and increases their antitumor cytotoxic program in the bone allowing the control of the tumor progression.

ERRα expression leads to high levels of CCL17 and CCL20 production in breast cancer cells

The aforementioned data strongly suggest that the expression of ERα by breast cancer cells influences the immune microenvironment to favor an efficient antitumor response. Interestingly, we failed to find any difference in Ki67 staining between CD8⁺ T cells evolving with either 4T1-CT or 4T1-ERRα bone metastasis (Supplementary Fig. 4A), suggesting that ERα expression affected T-cell recruitment to the bone rather than their proliferation in the bone. In order to address this hypothesis, we monitored the expression levels of several chemokines known to influence T-cell chemoattraction and found a 2- and 3-fold upregulation of Ccl17 and Ccl20 in 4T1-ERRα cells, respectively (Fig. 5A and B; Supplementary Fig. S5A and Supplementary Table S2). C29 or XCT-790 were sufficient to inhibit the overexpression of both Ccl17 and Ccl20, whereas no effect was observed in 4T1-ERRαAf2 cells ruling out any off-target effects (Fig. 5C and D; Supplementary Fig. S5B and S5C; ref. 26), arguing in favor of a direct role for ERα in the control of the expression of these two chemokines (13). Of note, the ability of ERα to upregulate Ccl17 and Ccl20 was also observed in other breast cancer cells including MCF7 and MDA-MB-231-B02 cells (Supplementary Fig. S5D–S5E; refs. 16, 18). Interestingly, the upregulation of Ccl17 and Ccl20, at both gene and protein levels due to ERα overexpression, was sustained in 4T1-ERRα bone metastasis (Fig. 5E and F) but lost in LM (Supplementary Fig. S5F). These data were reinforced following the analysis of ChIP-seq data revealing binding site for ERα in the promoter of Ccl17 and in Ccl20 (Supplementary Fig. S6A–S6D).

Given that CCL17 and CCL20 were reported to attract the fraction of activated CD8⁺ T cells expressingCCR4 and CCR6 (27), we next monitored the expression of both chemokine receptors on CD8⁺ T cells from the bone of animals bearing bone metastasis. In total agreement with the ability of 4T1-ERRα cells to sustain their production of CCL17 and CCL20 in the bone, and the activated phenotype of CD8⁺ T cells (Fig. 3), we found that, in bone colonized by 4T1-ERRα, a large fraction of CD8⁺ T cells expressed either CCR4 or CCR6 or both (Fig. 5G). Thus, breast cancer cells overexpressing ERα are...
endowed with a unique ability to produce high amounts of CCL17 and CCL20 and efficiently recruit activated CD8+ T cells to the bone.

ERRα expression in breast cancer cells reduces their TGFβ3 production and decreases TGFβ signaling in bone metastasis

Because the cytotoxic program of CD8+ T cells was largely exacerbated in legs bearing 4T1-ERRα cells, we next assessed the mechanisms by which ERRα overexpression in breast cancer cells increased their cytotoxic function in the bone. To this end, we further investigated the connection between CCL17-CCL20 and ERRα (ESRRA) by choosing a global approach combining bioinformatic analyses of protein interaction networks and transcriptional regulator databases (Supplementary Fig. S1, extended network, https://doi.org/10.18119/N9W891; Supplementary Fig. S2, minimal network, https://doi.org/10.18119/N9RK5C; ref. 28). We created the “Minimal Network specific to immune response to tumor” (containing 52 proteins; Fig. 6A), and by shortest path analysis, we then identified two new ESRRA-CCL17 or ESRRA-CCL20–associated regulators: VCAM and TGFβ3 (Fig. 6B and C). We and others reported that TGFβ signaling in T cells inhibits the cytotoxic differentiation program of CD8+ T cells both in humans and mice (29, 30), and we thus focused on this cytokine. The analysis of Tgfβ3 expression revealed a 70% decrease in 4T1-ERRα compared with 4T1-CT cells (Fig. 6D). Ex vivo bone cultures confirmed that ERRα upregulation in breast cancer cells negatively regulates Tgfb3 expression with a 55% decrease compared with control bone metastasis (Fig. 6E). Strikingly, immunohistological staining revealed that the production of TGFβ3 was largely decreased in breast cancer bone metastasis overexpressing ERRα (Fig. 6F). In agreement
Figure 2.
Overexpression of ERRα in breast cancer cells has no impact on LM development. A, Histologic analysis assessment of lung of mice 15 days after cell injection and hematoxylin and eosin coloration. * metastases. B, Graph illustrates the numbers of LM counted on three representative sections at different levels of deepness (n = 6). C–F, Lungs from the same mice used to quantify bone metastasis (BM, Fig. 1) were crushed, and cells released were cultured for 2 weeks. C and E are representative pictures of LM colonies, and D and F are graphs that illustrate the mean of number LM colonies formed for all mice ± SEM (n = 6; Mann-Whitney, TB/STV: P > 0.05). G, Graph illustrates the mean ERRα expression in LM cell colonies after 2 weeks of culture. Semiquantitative PCR was performed in triplicate on n = 3 animals (unpaired t test, ***, P < 0.0001) and normalized against the ribosomal protein gene L32 (mean ± SEM).
Figure 3.
Overexpression of ERRα in breast cancer cells promotes CD8⁺ T cytotoxic function. Cell suspensions were prepared from metastatic legs (bone; A and C-F) or lungs (B and C) of mice (n = 4) inoculated with 4T1-CT or 4T1-ERRα cells. A and B, Flow cytometry contour plots of CD45⁺ hematopoietic cells, illustrating the proportion of T cells (CD3⁺), B cells (CD19⁺), and CD4/CD8 T cells and graphs representing the percentage of cells in the bone (A; mean ± SD; n = 4; Student’s t test; ***, P < 0.001; ****, P < 0.0001) and in the lungs (B). C, Flow cytometry contour plots illustrating the production of FasL, LAMP-1, granzyme A and B, and IFNγ in CD8⁺ T cells. Graphs demonstrate the percentage of cells (D) and their absolute numbers (E). F and G, Fluorescent micrographs of sections of 4T1-CT or 4T1-ERRα expressing bone after TUNEL assay and enlarged views of dashed rectangles. Red, apoptotic cells; blue, cell nucleus after DAPI staining. G and H, Scale bar, 50 μm. T, tumor; GP, growth plate with apoptotic hypertrophic chondrocytes indicated by white arrows; Granz, granzyme.
with the decrease of Tgfβ3 expression in 4T1-ERRα, we observed an increase in Tgfβ3 levels in 4T1-ERRαAF2 bone metastasis (Fig. 6D) and a 5-fold increase in Tgfβ3 expression after treatment of 4T1-ERRα cells with the inverse agonist XCT-790 (Fig. 6G). Similar results were observed in MCF7 and MDA-MB-231-B02 human cell lines (Fig. 6H), ruling out an effect restricted to mouse 4T1 cells. Altogether, these results reveal that the overexpression of ERRα represses the expression of TGFβ3 in breast cancer and could thus prevent the breast cancer bone metastasis from creating an immunosuppressive microenvironment provided by TGFβ signal activation in immune cells.

In order to unconditionally confirm that CD8⁺ T cell evolving in 4T1-ERRα colonized bone escape TGFβ signaling control, we next analyzed CD8⁺ T cells from metastatic legs for the phosphorylation of SMAD2/3 proteins, which translates specifically the TGFβ signaling.

Figure 4.
CD8⁺ T-cell depletion restores 4T1-ERRα bone metastasis progression. 4T1-ERRα cell-injected mice were treated with anti-CD8 antibodies every 2 days once osteolytic lesions occurred, i.e., at day 10 based on radiography. A, Flow cytometry analysis confirming the CD8⁺ T-cell depletion in the bones. B, Radiography illustrating representative osteolytic lesions 18 days after 4T1-ERRα cells' inoculation. The osteolytic regions are delimited by dash lines. C, Three-dimensional microCT reconstruction representation of tibiae in sagittal sections (C) and cross-sections (D) following the cutting of the bone along the plane, illustrated by the dashed lines in C. E, Graphs illustrate the mean of the % of bone volume/tissues volume ± SEM (Mann-Whitney, BV/TV: P = 0.0037).
Figure 5. Ccl17 and Ccl20 upregulation by ERRα in breast cancer cells. 

A, Heat map representative of cytokine, chemokine, and growth factor mRNA expression in 4T1-ERRα and 4T1-CT cells, normalized against Ppia, Actb, and Rplp0 expression. 

B–D, Graph illustrates the relative gene expression after RT-PCR was performed on triplicate samples and normalized against the ribosomal protein gene L32 (mean ± SEM). B, 4T1-ERRα, 4T1-CT, or 4T1-ERRαAF2 and 4T1-CT(AF2) cells were analyzed \( n = 2 \); ANOVA, \( P < 0.0001 \) for Ccl17 and Ccl20; unpaired t test, \( P = 0.0007 \) (Ccl17), \( P = 0.0005 \) (Ccl20) 4T1-ERRα in 4T1-ERRαAF2 versus 4T1-CT. 

C and D, 4T1-ERRα and 4T1-ERRαAF2 cells were cultured for 24 hours with the inverse agonist C29 (5 \( \mu \)mol/L; unpaired t test, \( P = 0.0005 \) and \( P = 0.0034 \) for Ccl17 and Ccl20, respectively). 

E, Tumor cell colonies obtained after crushing bones extracted from mice \( n = 3 \) bearing either 4T1-ERRα or 4T1-CT lesions and cultured during 4 weeks were analyzed (unpaired t test, \( P = 0.0059 \) (Ccl17), \( P < 0.0001 \) (Ccl20), and \( P < 0.0001 \) (ERRα))]. 

F, Immunostaining for Ccl17 and Ccl20 on bone section colonized by either 4T1-ERRα or 4T1-CT cells. Bar, 200 \( \mu \)m. T, tumor; GP, growth plate. 

G, Representative flow cytometry contour plot illustrating the expression of CCR4 and CCR6 on CD8+ T cells from bones colonized with either 4T1-ERRα or 4T1-CT cells. All in vitro and ex vivo analyses were repeated at least twice. Data are plotted as mean ± SEM.
Overexpression of ERRα in breast cancer inhibits Tgfβ3 expression and affects TGFβ signaling in CD8+ T cells. A, Overlay of the Minimal Network (containing 101 proteins) with extracted information from the Gene Ontology consortium of proteins involved in the immune system process, as well as to create “Minimal Network specific to immune response to tumor” (containing 52 proteins). B and C, Potential new regulators of ESRRα, including VCAM1, MACF1, HUR, and TGFβ3, were identified through systematic shortest path definition of cross-talks. D and E, Graphs illustrating the relative gene expression after RT-PCR was performed on triplicate samples and normalized against the ribosomal protein gene L32 (mean ± SEM). D, 4T1-ERRα, 4T1-ERRαAF2, 4T1-CT, and 4T1-CT(af2) cells were analyzed (ANOVA, P < 0.0001 and unpaired t test, P = 0.0009 for 4T1-ERRαAF2 versus 4T1-CT(af2)). E, Tumor cell colonies obtained after crushing bones extracted from mice (n = 3) bearing either 4T1-ERRα, 4T1-CT cells, or 4T1-CT(af2) and 4T1-ERRαAF2 cells. Bar, 200 μm. T, tumor; GP, growth plate. F, Immunostaining for Tgfβ3 on bone section colonized by either 4T1-ERRα, 4T1-CT cells, or 4T1-CT(af2) and 4T1-ERRαAF2 cells. Bar, 200 μm. T, tumor; GP, growth plate. G, 4T1-ERRα cells were cultured for 24 hours with the inverse agonist XCT-790 (1 μmol/L; unpaired t test, P = 0.0007). Data are plotted as mean ± SEM. H, RT-PCR was performed on mRNA of MCF7 and B02 clones. Mean of three different cultures for each MCF7 clones is shown for TGFβ3 (unpaired t test, P = 0.0218 MCF7-ERRα versus MCF7-CT; B02 clones. ANOVA, P = 0.0027; unpaired t test, P = 0.007: B02-ERRα versus B02-CT). I, Representative flow cytometry histogram plot illustrating the phosphorylation of SMAD2/3 in CD8+ T cells from bones colonized with either 4T1-ERRα cells or 4T1-CT. The percentage of positive cells is illustrated. These ex vivo data are representative of two experiments with 3 to 4 mice per group. All in vitro analyses were repeated at least twice.
Table 1. Correlation in clinics in patients with breast cancer.

A

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<th>Correlation with ESRRA</th>
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<td>CCL17</td>
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<td>-0.215</td>
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B

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<th>Correlation with ESRRA</th>
<th>All (N = 110)</th>
<th>No mets (N = 50)</th>
<th>Visceral + bone (N = 53)</th>
<th>Bone only (N = 16)</th>
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<td>P value</td>
<td>R</td>
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<td>0.0001</td>
<td>-0.327</td>
<td>0.0201</td>
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Note: Meta-analysis of public datasets (GSE12276-GSE2034-GSE2603; n = 248) revealed a positive correlation between the expression of ESRRA and CCL17 and CCL20 and a negative correlation with TGFβ3 expression levels in luminal (A) and triple-negative (B) breast tumors; correlation scores were calculated using the Pearson correlation coefficient. P values less than 0.05 were considered statistically significant and are in bold.

activation (31). Clearly, SMAD2/3 phosphorylation was 2 to 3 times lower compared with that of CD8⁺ T cells from 4T1-CT metastatic legs (Fig. 6). Thus, in addition to increasing CD8⁺ T-cell recruitment to the bone, ERRα overexpression in breast cancer cells impairs their ability to produce high amounts of TGFβ3, decreasing TGFβ signaling in CD8⁺ T cells, a key repressor of their cytotoxic activity and capacity to eliminate cancer cells.

Overexpression of ESRRA (ERRα) in patient tumors is associated with high levels of CCL17, CCL20, and low levels of TGFβ3 expression

Finally, we addressed the relevance of our data obtained in mice to the human pathology. We performed a meta-analysis on 248 patients including luminal and triple-negative breast tumors (TNBC) split into four groups: all tumors (n = 248), patients without metastases (No Mts, n = 121), patients that had visceral and bone metastases (Visceral+Bone metastases, n = 53), and patients that had only bone metastases (Bone Only, n = 74). As in mice, the ESRRA (ERRα) expression was positively correlated with that of CCL17 and CCL20 and inversely proportional to that of TGFβ3 in patients with metastases restricted to the bone (Bone Only) with luminal and TNBC tumors (Table 1A and B). Correlations were also identified in No Mts, All, and Visceral + Bone groups of luminal or TNBC patients (Table 1A and B). In addition, ERRα expression was not associated with LM in two cohorts of patients with breast cancer (16). Thus, this set of data from human sample analyses strongly suggests that, similarly to mice, the overexpression of ERRα in human breast cancer cells allows them to create an immune-efficient environment in the bone by increasing the production of chemokines capable of attracting activated T cells to the bone and decreasing the production of TGFβ essential for repressing the cytotoxic activity of T cells.

Discussion

Cancer cells adapt to the microenvironment, shaped by their own doing, which in turn influences their fate. This interplay is particularly important for cells forming metastases, which leave their primary microenvironment to settle in a new, second one. Here, we revealed that the level of ERRα expression on breast cancer metastases promotes their ability to condition an efficient antitumor CD8⁺ T-cell response selectively in the bone.

CD8⁺ T cells have been described as critical inhibitors of bone metastases. Indeed, in mice, the alteration of CD8⁺ T-cell development after metastases implantation in the bone, or the deprivation of CD8⁺ T cells, was reported to increase tumor growth (5, 6). Osteoclasts have been depicted to secrete chemokines that can attract CD8⁺ T cells (32). However, the regulation of the bone metastasis burden by CD8⁺ T cells seems totally independent of the osteoclast activity (6). Our study reveals that the cancer cells per se can influence both the recruitment and the cytotoxic activity of the CD8⁺ T cells in the bone. Moreover, the ability of the breast cancer metastases to condition the immune response in the bone can be in part orchestrated by the levels of expression ERRα on the breast cancer and potentially to the sensitivity of metastases to the ERRα ligand(s). The selective effects of ERRα expression in breast cancer on the tumor burden of bone metastasis and antitumor response in the bone strongly suggest that unlike the lung, the bone could constitute a microenvironment with high levels of the ERRα ligand(s) that so far remain uncharacterized. Another alternative is that the lung, but not the bone, could be highly enriched in inhibitors of ERRα signaling or negative regulators of ERRα expression that remain to be identified. Thus, the ability of the metastases to induce or not a potent immune response may dictate by both the tumor per se and the microenvironment where it is anchored. In the case of breast cancer cells, we propose to place ERRα at the core of this interplay between metastases and their new microenvironment.

In addition to increasing the recruitment of activated CD8⁺ T cells to the bone, the overexpression of ERRα on breast cancer cells also decreased their ability to produce high amounts of TGFβ3. Depletion experiments confirmed that CD8⁺ T cells are key antitumor immune cells whose activation and recruitment are controlled by the levels of ERRα expression on bone metastasis. All forms of TGFβ have been reported as potent immune-regulators and share a common receptor (33). Although TGFβ1 is predominant in the immune system, TGFβ3 is mainly produced by muscles, bones but also by various cancer cells (34). The repression of TGFβ3 production in ERRα breast
cancer cells subsequent affects TGFβ signaling in CD8+ T cells present in the bone. TGFβ signaling represses the expression of numerous transcription factors associated with cytotoxicity, as well as T-Bet, a key inducer of IFNγ (35). Therefore, overexpressed ERRα breast cancer cells that settle in the bone are unable to sustain an immunosuppressive microenvironment based on high levels of TGFβ signaling in T cells and repression of cytotoxic program and IFNγ production. Interestingly, IFNγ also contributes to the suppression of bone metastasis. Indeed, IFNγ has been reported to reduce both RANKL expression and osteoclast formation, counterbalancing the aberrant bone resorption, which facilitates tumor growth (36). Concomitantly, inhibition of bone resorption also leads to the decrease in TGFβ release from the bone matrix (4), thus potentially contributing to amplifying the activation of T cells including their production of IFNγ.

It is likely that effector CD8+ T cells that reach the bone metastases have previously been primed in the draining lymph nodes or by the spleen-presenting antigens from the primary tumor and/or the metastases. As in the primary tumor, the activated CD8+ T-cell population in contact with the bone metastasis is actually heterogeneous and composed of cells recently activated and activated memory cells. Interestingly, in both mice and humans, the fraction of CD8+ T cells that expresses CCR6 and CCR4 has been depicted to rapidly mount an efficient response, corresponding to activated/memory T cells (27). Once implanted in the bone, we found that the breast cancer overexpressing ERRα has unique ability to sustain high expression of CCL17 and CCL20 and low expression of TGFβ3, thus attracting the activated/memory CD8+ T cells whose anti-tumour cytotoxic function is magnified by the lack of repression by TGFβ signaling.

In conclusion, this study assigns an unsuspected role for ERRα expression in breast cancer on the bone immune system that conditions the bone metastasis growth outcome, providing the mechanistic basis for understanding how ERRα expression in breast cancer can affect the bone microenvironment and reduce bone metastasis growth. ERRα seems to appear at the core of this interplay between breast cancer metastases and their new environment, integrating signals from the microenvironment to develop an efficient anti-tumour response. Therefore, we propose to consider ERRα expression on breast cancer as a biomarker predictive of bone metastasis response to immunotherapy and/or as a good prognosis marker in bone metastasis progression once established, opening the path toward to the clinical use of ERRα agonist to relieve patients with ERRα-positive bone metastasis after primary tumor resection.

Disclosure of Potential Conflicts of Interest
P. Clézardin reports receiving honoraria from the speakers’ bureau of Amgen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: J.C. Marie, E. Bonneyle
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Bouchet, A. Lainé, M. Proponnet-Guerault, L. Bouazza, S. Geraci, S. El-Moghrabi, M. Duterque-Coquillaud, J.C. Marie, E. Bonneyle
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Bouchet, A. Lainé, C. Boyault, E. Meugnier, C.W.S. Kan, H. Hernandez-Vargas, M. Duterque-Coquillaud, J.C. Marie, E. Bonneyle
Writing, review, and/or revision of the manuscript: A. Lainé, C. Boyault, H. Hernandez-Vargas, Y. Yoshiko, M. Duterque-Coquillaud, P. Clézardin, J.C. Marie, E. Bonneyle
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Bouchet, M. Proponnet-Guerault, M. Duterque-Coquillaud, J.C. Marie, E. Bonneyle
Study supervision: J.C. Marie, E. Bonneyle

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References

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A. Lainé
Writing, review, and/or revision of the manuscript:
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Bouchet, M. Proponnet-Guerault, M. Duterque-Coquillaud, J.C. Marie, E. Bonneyle
Study supervision: J.C. Marie, E. Bonneyle

Contributions
J.C. Marie, E. Bonneyle
M. Bouchet
L. Bouazza
S. Geraci
S. El-Moghrabi
M. Duterque-Coquillaud
J.C. Marie
E. Bonneyle
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