

Extratumoral Insulin-Like Growth Factor-1 Promotes Resistance To HER2-Targeted Tyrosine Kinase Inhibitors And Macrophage Polarization Through Redox Signaling

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Article

Keywords: HER2-positive breast cancer, IGF-1 signaling, PI3K–NRF2 pathway, Oxidative stress, Tumor microenvironment, Macrophage polarization

Posted Date: October 18th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11070146/v1>

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Additional Declarations: No competing interests reported.

Abstract

Adaptive resistance to HER2-targeted tyrosine kinase inhibitors (TKIs) such as lapatinib and tucatinib limits durable responses in HER2-positive breast cancer. While tumor-intrinsic resistance mechanisms are well studied, the role of extratumoral cues in therapy adaptation remains unclear. Here we demonstrate that extratumoral insulin-like growth factor-1 (IGF-1) is a critical mediator of adaptive TKI resistance. TKI treatment induces oxidative stress, marked by elevated mitochondrial reactive oxygen species (ROS), lipid peroxidation, and DNA damage. Single-cell analysis and fibroblast co-culture experiments identify stromal fibroblasts as the primary source of this IGF-1. This stromal IGF-1 activates a PI3K/FOXO/NRF2 axis in TKI-stressed tumor cells that suppresses KEAP1, stabilizes NRF2, and restores redox homeostasis, allowing cancer cells to persist. Inhibiting IGF-1R or PI3K blocks NRF2 activation and restores TKI sensitivity, making this a targetable resistance mechanism. IGF-1 signaling also remodels the tumor secretome and drives macrophage polarization toward an immunosuppressive M2 phenotype, further supporting tumor survival. Consistent with this, residual tumors from HER2-TKI-treated patients showed upregulated IGF-1R signaling and a partial antioxidant gene signature. IGF-1/PI3K/NRF2 pathway activity correlated with M2 macrophage infiltration across TCGA breast cancer cohorts. Together, these findings identify a pharmacologically actionable, microenvironment-driven redox adaptation axis that links tumor cell survival to immune modulation and suggest that co-targeting IGF-1/PI3K/NRF2 signaling could improve the efficacy of HER2-targeted therapy. Durable responses to targeted therapy may depend not only on disrupting tumor-intrinsic signaling but also on the stromal and immune microenvironment tumors co-opt to survive treatment.

Introduction

Receptor tyrosine kinase inhibitors (TKIs), including lapatinib, neratinib, and tucatinib, have significantly improved clinical management and overall prognosis in HER2-positive breast cancers. However, acquired resistance to HER2-targeted TKIs remains a major clinical challenge, often leading to disease recurrence and limiting long-term therapeutic benefit. Therefore, understanding the dynamic molecular mechanisms underlying acquired resistance is essential to developing more durable and effective treatment strategies ¹.

Historically, studies of TKI resistance have primarily focused on tumor cell-intrinsic mechanisms. Among these, compensatory activation of the Insulin-like Growth Factor-1 Receptor (IGF-1R) pathway is a well-established mechanism that sustains Phosphatidylinositol 3-kinase/Protein Kinase B (PI3K/AKT)-dependent survival signaling following HER2 inhibition ². However, these classical resistance models largely interpret IGF-1R activation as an autocrine and tumor cell-restricted process driven by receptor crosstalk, pathway amplification, or intrinsic feedback signaling ³. Resistance can also arise non-cell-autonomously: fibroblast-secreted factors have been shown to sustain mTOR/AKT signaling and blunt lapatinib sensitivity in HER2-positive breast cancer cells, independent of tumor-intrinsic alterations ⁴. Stromal components, particularly cancer-associated fibroblasts, are a major source of IGF-1 in the

mammary gland ⁵. However, the functional role of extratumoral IGF-1 in resistance to HER2-targeted therapy remains poorly understood. Specifically, it is unclear whether the TME merely provides a constitutive supply of prosurvival growth factors or whether HER2-targeted TKIs actively induce adaptive stromal support that enables tumor cells to survive pharmacological stress.

In addition to suppressing HER2/PI3K-driven proliferative signaling, TKIs such as lapatinib also disrupt cellular redox homeostasis by inducing reactive oxygen species (ROS) ^{6,7}. While excessive oxidative stress promotes cell death, moderate ROS accumulation can function as a secondary signaling mechanism that activates adaptive survival pathways ^{8,9}. Under physiological conditions, IGF-1 signaling protects normal tissues from oxidative injury by activating NRF2 (Nuclear Factor Erythroid 2-Related Factor 2)-dependent antioxidant programs that maintain redox homeostasis ¹⁰. However, whether breast tumors exploit TME-derived IGF-1 to establish a similar antioxidant state and evade TKI-induced oxidative stress remains unknown. Moreover, because tumor-secreted factors can shape the polarization of surrounding immune cells, it remains unclear whether this stromal-tumor antioxidant crosstalk also extends to remodeling the immune microenvironment, potentially influencing macrophage-mediated tumor clearance ¹¹.

We hypothesize that HER2-targeted TKIs, including lapatinib and tucatinib, mobilize stroma-derived IGF-1 to confer a non-canonical cytoprotective effect in HER2-positive breast cancer cells. We further propose that paracrine IGF-1 signaling promotes resistance to TKI-induced oxidative stress by activating the PI3K/NRF2 antioxidant axis, thereby enabling adaptive tumor cell survival and reshaping macrophage-mediated immune responses within the tumor microenvironment. Accordingly, this study aimed to define the functional crosstalk between stromal IGF-1 signaling and intracellular PI3K/NRF2-mediated redox adaptation in acquired HER2 TKI resistance and macrophage polarization.

Results

Residual disease following HER2 TKI lapatinib therapy reveals a stress-adapted transcriptional state.

To characterize early responses to HER2 therapy, we analyzed transcriptomic profiles from HER2-positive breast cancer patients treated with lapatinib (GSE130788), comparing residual disease (RD) samples collected after 14 days with pre-treatment biopsies (Supplemental Tables S1 and S2). Pathway analysis (MSigDB, Reactome, KEGG, WikiPathways) showed suppression of cell-cycle progression genes (E2F targets, G2/M checkpoint) and inflammatory/NF- κ B signaling (Inflammatory Response, TNF α Signaling via NF- κ B) in RD samples, along with upregulation of a cellular senescence signature (Fig. 1A). These findings indicate that RD cells exit active proliferation and inflammation and adopt a growth-arrested state.

The senescence signature was largely derived from PI3K/AKT pathway genes (PTEN, PIK3CA, PIK3CB, PIK3R1, AKT2, AKT3, TSC2, MAPK1, MAPK3). Among the RTK-linked terms enriched in RD, IGF-1/IGF1R signaling was represented at multiple levels: IGF1R and INSR themselves, their adaptors IRS1/IRS2, and

downstream mTORC1 components (RPTOR, MLST8) were all captured within the enriched FOXO and mTOR signaling terms (Fig. 1A). Because this IGF-1/PI3K/AKT module was embedded in a growth-arrested rather than proliferative state, we inferred it was unlikely to drive proliferation in RD cells, a possibility we examined in subsequent experiments.

RD samples also showed a trend toward increased expression of xenobiotic metabolism genes, including cytochrome P450 and phase I/II drug-metabolizing enzymes, though this did not reach statistical significance after correction (Fig. 1A). CYP3A4-mediated oxidative metabolism of lapatinib generates reactive intermediates implicated in TKI-associated hepatotoxicity¹², and given IGF-1's established role in redox homeostasis¹⁰, we hypothesized that the non-proliferative IGF-1/IGF1R signature in RD reflects a stress-adaptive response to this metabolic burden rather than a proliferative function. Consistent with this, RD samples showed significant upregulation of IGF1R ($\log_2FC = 0.32$, $adj.p = 2.67 \times 10^{-4}$; $\log_2FC = 0.17$, $adj.p = 5.32 \times 10^{-6}$), while IGF1 showed a consistent but sub-significance-threshold upward trend across four probes ($\log_2FC$ range 0.17–0.28, $adj.p = 0.0505$ –0.185). Insulin (INS) was modestly decreased ($\log_2FC = -0.057$, $adj.p = 0.0179$) and INSR was unchanged ($adj.p = 0.117$), arguing against an INS-driven alternative to IGF1R activation. However, because these data came from bulk tumor biopsies, we could not determine whether tumor or stromal cells drove this signaling, so we examined the cellular source of IGF-1 within the tumor microenvironment.

Stroma is the primary source of paracrine IGF-1 in breast tumors.

We analyzed the relationship between IGF-1 expression and tumor composition in the TCGA breast cancer (BRCA) cohort using the TIMER2.0 web server. Across the pooled BRCA cohort and both molecular subtypes examined (HER2-enriched, LumB), IGF-1 expression consistently and significantly correlated negatively with tumor purity (Spearman's $\rho = -0.40$ to -0.54 , $p < 10^{-6}$ in all three), consistent with IGF-1 in breast tumors originating predominantly from the non-tumor-cell compartment rather than from the cancer cells themselves (Fig. 1B).

To validate this at single-cell resolution, we analyzed the BRCA_GSE114727_inDrop breast cancer scRNA-seq dataset (immune cells from eight breast cancers, matched normal breast tissue, blood, and lymph nodes, total 19676 cells)¹³ using TISCH2. IGF-1 expression was markedly enriched in stromal populations (fibroblasts and myofibroblasts) and showed minimal expression in immune compartments (Figs. 1C-D). In an additional analysis of eight breast cancer patients (six luminal-like and two basal-like; BRCA_GSE138536; total 1902 cells)¹⁴ comparing IGF-1 expression across epithelial cells, fibroblasts, mono/macrophages, and myofibroblasts, we observed the same fibroblast-restricted pattern, confirming fibroblast-specific IGF-1 expression (Figs. 1E-F). According to this dataset, IGF1R expression was restricted to epithelial cells, monocytes/macrophages, and myofibroblasts-cell types/compartments with nearly negligible expression of its ligand (Fig. 1G). These data confirm that stromal, rather than tumor, cells are the primary source of IGF-1 in the tumor microenvironment, shifting our focus from tumor-intrinsic to microenvironment-driven IGF-1 signaling.

Consistent with this stromal origin, gene-level analysis of the lapatinib RD transcriptome (Fig. 1A) identified specific upregulation of IGFBP4 and its cognate protease, PAPPA¹⁵ ($\log_2FC + 0.30$ and $+ 0.10$, $\text{adj.p} = 0.0012$ and 0.0013), suggesting that enhanced proteolytic release of bioavailable IGF-1 from this stromal reservoir may further potentiate microenvironment-driven signaling in residual disease. Additional IGFBP family members (IGFBP1, IGFBP6) and proteases (MMP7, KLK11, MMP2, CTSG) were also significantly upregulated in RD (Supplemental Tables S1 and S2), though a specific cognate binding protein-protease relationship was confirmed only for IGFBP4/PAPPA in this analysis.

Figure 1: Residual disease exhibits a stress-adaptation signature, with fibroblasts identified as the primary source of IGF-1. **A.** Publicly available gene expression data (GSE130788) from HER2-positive breast cancer patients treated with lapatinib (residual disease/RD vs pre-treatment biopsies) were analyzed using Enrichr, an interactive gene enrichment analysis tool. Top pathways enriched in the RD, as per MSigDB, Reactome, Wikipathway, and KEGG, are shown. **B.** The TCGA-BRCA, BRCA-Her2, and BRCA-LumB cohorts were queried via TIMER2.0 to assess the relationship between IGF-1 expression and tumor purity. **C-G.** The single-cell RNA sequencing datasets GSE114727_inDrop and GSE138536 from human breast cancer samples were queried via TISCH2 to compare IGF-1 expression densities across epithelial, stromal, and immune cell populations.

HER2 TKIs induce mitochondrial ROS and oxidative stress, impacting tumor cell survival.

HER2-TKI lapatinib, metabolized by CYP3A4/5, forms reactive compounds that generate ROS and cause liver damage^{12,16,17}. Tucatinib, a third-generation HER2 inhibitor, is hepatotoxic^{18–20}, likely due to ROS generation and similar metabolism. We chose the HER2-overexpressing breast cancer cell line MDA-MB-453²¹, previously used to study response to lapatinib²², to examine mitochondrial ROS production by lapatinib and tucatinib because it showed similar CYP3A5 mRNA upregulation within 6h of lapatinib and 12h of tucatinib treatment, like HepG2, a cell line used for studying liver metabolism²³ (Supplemental Figure S1).

To assess whether lapatinib and tucatinib induce oxidative stress in MDA-MB-453 cells, we measured ROS using MitoSOX Red and standardized their doses for later experiments (Supplemental Figure S2). Both increased mitochondrial ROS, which pre-treatment with the ROS scavenger N-acetylcysteine (NAC)²⁴ significantly reduced (Figs. 2A–B). Although CYP-mediated drug metabolism is classically studied in hepatocytes, CYP3A4 is expressed in 20–55% of breast cancer tissue, where it actively metabolizes chemotherapeutic agents *in situ*²⁵; however, comparable tissue-expression data for CYP3A5 in breast cancer are limited. We therefore hypothesized that the CYP3A5 upregulation we observed in MDA-MB-453 (Supplemental Figure S1) similarly supports local, metabolism-dependent ROS production analogous to the hepatic bioactivation pathway.

We assessed downstream effects of ROS production, such as lipid peroxidation, measured with BODIPY 581/591 C11, which increased after TKI treatment but was blocked by NAC (Figs. 2C–D). Mitochondrial

membrane potential, assessed by TMRM fluorescence, also decreased, indicating mitochondrial dysfunction due to enhanced ROS production, but NAC treatment restored it (Figs. 2E–F).

To assess if mitochondrial ROS causes genomic damage, we quantified nuclear 53BP1 foci, a DNA damage marker ²⁶. Both lapatinib and tucatinib significantly increased 53BP1 foci, an effect markedly reduced by NAC pre-treatment (Figs. 2G–H), indicating that ROS contributes to DNA damage. Together, these findings indicate increased oxidative stress in TKI-treated MDA-MB-453 cells.

We next studied how this TKI-induced oxidative stress impacted cell survival. Live-cell imaging showed a significant decrease in viable cells (calcein-AM) and increased cell death (PI) after TKI treatment, which NAC prevented (Figs. 2I–J).

In summary, HER2 TKIs lapatinib and tucatinib cause ROS-dependent oxidative stress in breast cancer cells, indicated by mitochondrial dysfunction, lipid peroxidation, and DNA damage, and reduce cell survival.

Figure 2: Lapatinib and tucatinib trigger reactive oxygen species production, DNA and mitochondrial damage and cytotoxicity in HER2-positive breast cancer cells, effects that N-Acetylcysteine (NAC) can counteract significantly. A & B. Assessment of mitochondrial ROS production. MDA-MB-453 cells were pretreated with or without 250µM NAC for 6h before exposing to 3.5µM lapatinib for 1h (**A**) and 5µM tucatinib for 36h (**B**). Live-cell images were captured at 20X magnification using an epifluorescence microscope. Blue channel represents Hoechst-stained nucleus and red represents MitoSOX, the mitochondrial ROS-indicator. Graphs show the quantification of mean signal intensity from multiple microscopic images. **C & D.** Assessment of lipid peroxidation. Lipid peroxidation was measured in MDA-MB-453 cells after 12h treatment with 3.5µM lapatinib (**C**) and 5µM tucatinib (**D**), pretreated with NAC as mentioned above. Images were captured at 20X (2X zoom) using a confocal microscope. Graphs represent the quantification of average signal intensity of oxidized (red) to reduced (green) C11-BODIPY staining. **E & F.** Assessment of mitochondrial membrane potential. Mitochondrial damage was assessed by measuring loss of mitochondrial membrane potential in lapatinib (**E**) and tucatinib (**F**)-treated MDA-MB-453 cells (as above) using TMRM (red), counterstained with Hoechst (blue). Live-cell images were captured at 20X using an epifluorescence microscope. **G & H.** Assessment of 53BP1 expression. Expression of the DNA damage repair protein 53BP1 was measured by immunofluorescence with an anti-53BP1 antibody (red) after lapatinib (**G**) and tucatinib (**H**) treatment with or without prior treatment with NAC as indicated above. Cells were counterstained with nuclear dye DAPI (blue), and images were captured at 100X with a confocal microscope. **I & J.** Assessment of cell viability and death. Live and dead cell numbers were measured after 48h of lapatinib (**I**) or tucatinib (**J**) treatment, with or without NAC pretreatment as mentioned above. Viable and dead cells were stained with calcein AM (green) and propidium iodide/PI (red), respectively. Images were captured at 20X in a confocal microscope. The bar graphs show the quantification of the average number of live and dead cells.

Experiments were repeated up to three times with biological replicates. Data points represent the average of three replicates with standard error (SE). The p-values for the different comparisons are listed

in Supplemental Table 5.

IGF-1/IGF-1R signaling counteracts TKI-induced oxidative stress and promotes tumor cell survival.

After establishing that HER2 TKIs cause oxidative stress, we examined how tumor cells withstand it, especially through IGF-1 signaling. While IGF-1 signaling is known to promote resistance to HER2-targeted treatments^{3,27,28}, its role in reducing therapy-induced oxidative stress remains uncertain. We hypothesized that IGF-1 protects cells by inhibiting TKI-induced ROS, consistent with its oxidative-stress protective functions¹⁰.

We pretreated MDA-MB-453 cells with exogenous IGF-1 before TKI exposure. To do so, we first standardized the optimal IGF-1 dose and exposure duration (Supplemental Figure S3). IGF-1 notably decreased mitochondrial ROS levels after TKI treatment, as shown by live-cell imaging, and this reduction was reversed by siRNA knockdown of IGF-1R (Figs. 3A–C). This indicates that IGF-1/IGF-1R signaling is required for ROS suppression.

Consistent with this, we found that recombinant IGF-1 consistently improved cell survival after TKI, but IGF-1R depletion reversed this protection (Figs. 3D–E). Linsitinib, an IGF-1R/INSR dual inhibitor, prevented ROS suppression and survival benefits, mimicking these effects (Figs. 3F–I). Together, these findings confirm that IGF-1/IGF-1R signaling is protective against TKI-induced oxidative stress.

TKI-induced oxidative stress temporally coincides with dynamic changes in IGF-1/IGF-1R signaling.

To explore the temporal relationship between TKI-induced oxidative stress and IGF-1 signaling, we performed time-course experiments after TKI treatment. Mitochondrial ROS levels increased after TKI exposure, coinciding with increased IGF-1 and IGF-1R transcripts. As ROS levels decreased, the IGF-1 pathway also declined (Figs. 3J–K). Correlation analysis revealed a moderate-to-strong link between mitochondrial ROS and IGF-1/IGF-1R expression (Fig. 3L and Supplemental Figure S4). Combined with our earlier finding that IGF-1/IGF-1R signaling is required for ROS suppression (Figs. 3A–C), these results are consistent with a stress-responsive feedback loop in which TKI-induced ROS transiently activates IGF-1/IGF-1R signaling, which in turn limits further ROS accumulation.

IGF-1 induces a sustained redox-adapted state with context-dependent vulnerabilities.

To determine whether IGF-1 protection causes long-term phenotypic changes, we conducted rechallenge experiments 15 days after cells recovered from TKI exposure. Surviving cells showed decreased ROS levels upon re-exposure to baseline TKI doses, indicating a stable redox-adapted state (Fig. 3M). Interestingly, prior IGF-1 exposure increased resistance to oxidative stress, as indicated by reduced ROS production after doxorubicin treatment (Fig. 3M), demonstrating induction of a broad antioxidant program. This adaptive state also caused vulnerabilities. Cells preconditioned with IGF-1 survived better with tucatinib, showing preserved antioxidant defenses (Fig. 3N). However, high-dose lapatinib almost completely eradicated IGF-1-preconditioned cells, more than cells that developed resistance to lapatinib

alone without prior preconditioning (Fig. 3N). This differential response likely stems from lapatinib's broader target profile, including EGFR, HER2, and HER4²⁹; by inhibiting these additional receptors, lapatinib may disrupt compensatory signaling needed to sustain IGF-1-dependent antioxidant defenses. These findings suggest IGF-1-driven adaptation creates a vulnerability with dual ErbB inhibition.

These data demonstrate IGF-1 signaling helps tumor cells survive TKI-induced oxidative stress by establishing a redox-adapted state. This adaptation confers broad resistance to oxidative stressors but creates a selective vulnerability to therapies that co-inhibit EGFR/HER4 alongside HER2.

Figure 3. IGF-1 protects cells from lapatinib and tucatinib-induced ROS production and cytotoxicity through IGF1-R in HER2-positive breast cancer cells. **A.** Assessment of siRNA knockdown of IGF-1R. RT-qPCR confirms IGF-1R knockdown efficiency in MDA-MB-453 cells. **B & C.** Assessment of lapatinib and tucatinib-mediated mitochondrial ROS production after IGF-1R knockdown. MDA-MB-453 cells were pretreated with 50ng/ml IGF-1 for 6h before exposing them to 3.5μM lapatinib for 1h (**B**) and 5μM tucatinib for 36h (**C**). **D & E.** Assessment of lapatinib and tucatinib-mediated cytotoxicity after IGF-1R knockdown. Following 6h of pretreatment with IGF-1, MDA-MB-453 cells were exposed to 3.5μM lapatinib (**D**) and 5μM tucatinib (**E**) for 48h before measuring cytotoxicity with calcein-AM and PI dyes. The numbers of viable (calcein) and dead (PI) cells were counted and graphically represented. **F & G.** Assessment of lapatinib and tucatinib-mediated mitochondrial ROS production after pharmacological inhibition of IGF-1R. MDA-MB-453 cells were pretreated with IGF-1R/INSR inhibitor linsitinib (40μM) and IGF-1 (50ng/ml) for 6h and then exposed to 3.5μM lapatinib for 1h (**F**) and 5μM tucatinib for 36h (**G**). Mitochondrial ROS were measured via live-cell imaging with the MitoSOX dye, as previously described. **H & I.** Measurement of cytotoxicity after pharmacological inhibition of IGF-1 followed by lapatinib and tucatinib treatment. Following co-pretreatment, MDA-MB-453 cells were exposed to lapatinib (**H**) and tucatinib (**I**) for 48h. Viable and dead cells were measured via calcein-AM and PI staining. **J & K.** Evaluation of correlation between mitochondrial ROS production, IGF-1 and IGF-1R expression by HER2-positive breast cancer cells after lapatinib and tucatinib treatment. Mitochondrial ROS generation was measured in MDA-MB-453 cells after 3.5μM lapatinib (**J**) and 5μM (**K**) tucatinib treatment at different time points (lapatinib- 0, 1, 3, 6, 10, and 24h and tucatinib- 0, 12, 24, 36, 40, and 48h). Simultaneously, IGF-1 and IGF1-R expressions were measured at these time points via RT-qPCR, normalized against actin. **L.** Correlation between drug-induced mitochondrial ROS, IGF-1 and IGF-1R expressions using Python code. **M & N.** Exploring persistent eco-evolutionary memory against drug-induced oxidative stress following transient IGF-1-mediated rescue. MDA-MB-453 cells surviving the initial 48h TKI exposure (with or without 6h IGF-1 pretreatment) were isolated and cultured in standard drug-free conditions. Following a 15-day recovery, equal numbers of persister cells from both IGF-1-pre-treated and untreated TKI-treated groups were rechallenged in serum-free media with either lapatinib/tucatinib or doxorubicin. Mitochondrial ROS generation (**M**) and cytotoxicity (**N**) were measured to quantify the stable, broad-spectrum antioxidant capacity conferred by prior transient IGF-1 exposure.

Experiments were repeated up to three times with biological triplicates. Data points represent the average of three replicates with standard error (SE). The p-values among different comparisons are

included in the Supplemental Table 6.

Pharmacological inhibition of PI3K/NRF2 abolishes IGF-1-mediated ROS suppression.

The role of IGF-1 in overcoming therapeutic resistance is well recognized, primarily through anti-apoptotic signals downstream of PI3K/AKT activation³⁰. However, transcriptomic analysis of HER2-positive residual disease (RD) revealed suppression of key proliferative and survival pathways during the early adaptive phase after HER2 TKI treatment (Fig. 1), suggesting that persister cell survival relies on alternative stress-adaptive mechanisms. Given IGF-1's known role in redox balance via PI3K/AKT/NRF2 signaling in vascular and neuronal systems¹⁰, we hypothesized that IGF-1's protection against TKI-induced oxidative stress also depends on the PI3K/NRF2 pathway. To test this hypothesis, we blocked PI3K and NRF2 functions with BEZ-235, a dual PI3K/mTOR inhibitor³¹, and camptothecin (CT), an NRF2 transcriptional inhibitor³². After optimizing the dosage (Supplemental Figure S5), MDA-MB-453 cells were pretreated with IGF-1 (50ng/ml) alone or combined with either BEZ-235 (100nM) or CT (250nM) for 6h before exposure to lapatinib (3.5μM) or tucatinib (5μM). Live-cell imaging showed that blocking either PI3K or NRF2 prevented IGF-1 from neutralizing ROS, leading to a significant increase in mitochondrial ROS following TKI treatment (Figs. 4A-B). Additionally, calcein-AM/PI staining conducted 48h post-treatment revealed that disrupting the PI3K/NRF2 pathway significantly diminished IGF-1's cytoprotective effect and increased cell death (Figs. 4C-D).

Genetic depletion of NRF2 confirms NRF2-dependent cytoprotection.

Since camptothecin (CT) targets DNA topoisomerase I and isn't exclusive to NRF2 inhibition, we used NRF2-specific siRNA to deplete NRF2 (Fig. 4E). The NRF2-depleted cells underwent the same IGF-1 pretreatment and TKI exposure. As with pharmacological inhibition, NRF2 knockdown reduced IGF-1's protective effect, resulting in higher mitochondrial ROS levels and more cell death after lapatinib and tucatinib (Figs. 4F-I).

IGF-1 enhances NRF2 activation and antioxidant gene expression.

To assess whether IGF-1 directly activates NRF2-dependent antioxidant signaling, we measured nuclear localization and transcript levels of NRF2 and its downstream targets NQO1, HMOX1, and GSR. MDA-MB-453 cells were pretreated with IGF-1 alone or combined with the IGF-1R inhibitor linsitinib before TKI exposure. Immunofluorescence showed increased nuclear retention of NRF2 after IGF-1 pretreatment in TKI-treated cells, an effect blocked by linsitinib (Figs. 4L-M). Consistent with this, IGF-1 pre-treatment enhanced expression of NRF2 target genes in an IGF-1-dependent manner, though the magnitude and statistical significance varied by TKI and target gene (Figs. 4J-K).

Given the strong link between mitochondrial ROS accumulation and IGF-1/IGF-1R signaling (Figs. 3J-L), we examined whether NRF2 activation followed a similar pattern. Time-course studies showed nuclear NRF2 levels closely followed mitochondrial ROS production and IGF-1/IGF-1R increases after TKI

treatment (Figs. 4N-Q). Correlation analysis confirmed a strong relationship among nuclear NRF2, mitochondrial ROS, and transient IGF-1 signaling activation (Fig. 4R; Supplemental Figure S6).

These findings suggest TKI-induced oxidative stress triggers an adaptive response, with IGF-1-dependent NRF2 stabilization contributing to redox balance restoration, though with distinct kinetics between lapatinib and tucatinib.

FOXO suppression links IGF-1/PI3K signaling to NRF2 stabilization.

Multiple studies show an inverse relationship between NRF2 and FOXO1/3, with FOXO inactivation promoting NRF2 activation^{33–35}. Because PI3K/AKT signaling inhibits FOXO1/3 through phosphorylation-dependent cytoplasmic retention³⁶, we hypothesized that IGF-1–induced NRF2 activation involves suppression of FOXO1/3 via AKT activation. We examined phosphorylated FOXO1/3 by immunoblotting at 24h. We chose this time point because compensatory RTK feedback following HER2 TKI treatment is well established by this stage: FOXO3a-dependent HER3 upregulation begins at 4h, with recovery of receptor phosphorylation detectable by 13 h and sustained through 48h³⁷. Similarly, FoxO-dependent induction of IGF-1R and INSR, with corresponding phosphorylation changes, is established by 24h after PI3K/AKT pathway inhibition²².

MDA-MB-453 cells were pretreated with IGF-1 alone or with linsitinib before TKI exposure. Both TKIs increased pFOXO1/3 levels (lane 3 vs. 1). This is consistent with compensatory autocrine IGF-1/IGF-1R activation following TKI treatment (Figs. 3J-L). IGF-1 pretreatment sustained this increase (lane 4 vs. 3). Conversely, linsitinib, added with exogenous IGF-1 and TKI, decreased FOXO1/3 phosphorylation below TKI-alone levels (lane 5 vs. lane 3) (Figs. 4S-T). These results support the conclusion that IGF-1 signaling suppresses FOXO1/3 activity during TKI-induced oxidative stress.

KEAP1 is both a negative regulator of NRF2 and a FOXO1/3 target³⁵. We therefore examined KEAP1 and NRF2 transcript levels under identical experimental conditions (Supplemental Figure S7). IGF-1 alone decreased KEAP1, even without TKI treatment. This suggests a direct IGF-1/AKT/FOXO-mediated KEAP1 repression. TKI treatment alone similarly decreased KEAP1 and modestly increased NRF2, paralleling the pFOXO1/3 pattern. IGF-1 pretreatment further enhanced NRF2 induction in TKI-treated cells, and linsitinib restored both NRF2 and KEAP1 toward baseline levels, which were not significantly different from untreated controls, consistent with IGF-1R-dependent regulation of both targets (Supplemental Figure S7).

Together, these results suggest that IGF-1/IGF1R signaling promotes NRF2 activation by suppressing FOXO1/3 via PI3K/AKT, thereby reducing KEAP1.

Figure 4. IGF-1 protects HER2-positive breast cancer cells from lapatinib and tucatinib-induced oxidative stress through the PI3K/NRF2 pathway. A & B. Assessment of lapatinib and tucatinib-induced ROS after pharmacological inhibition of PI3K and NRF2. MDA-MB-453 cells were pretreated with 50ng/ml IGF-1 with or without (100nM) BEZ-235-PI3K inhibitor and (250nM) Camptothecin (CT)-NRF2

inhibitor for 6h before exposing them to 3.5 μ M lapatinib for 1h (**A**) and 5 μ M tucatinib for 36h (**B**). **C & D**. Assessment of lapatinib and tucatinib-induced cytotoxicity after pharmacological inhibition of PI3K and NRF2. Following pretreatment with IGF-1 with or without BEZ-235 and CT for 6h (as above), MDA-MB-453 cells were exposed to lapatinib (**C**) and tucatinib (**D**) for 48h to measure cytotoxicity with calcein-AM (live cell) and PI (dead cell) dyes. **E**. Assessment of siRNA-mediated knockdown of NRF2. RT-qPCR confirming NRF2 knockdown efficiency in MDA-MB-453 cells. **F & G**. Measurement of mitochondrial ROS after genetic ablation of NRF2 followed by lapatinib and tucatinib treatment. Following NRF2 RNAi, MDA-MB-453 cells were first exposed to IGF-1, followed by lapatinib (**F**) and tucatinib (**G**) treatment. **H & I**. Evaluation of lapatinib and tucatinib-mediated cytotoxicity following NRF2 knockdown. After pretreatment with IGF-1, MDA-MB-453 cells were exposed to (**H**) lapatinib and (**I**) tucatinib for 48h to measure cytotoxicity. **J & K**. Measurement of transcript levels of NRF2 targets after lapatinib and tucatinib treatment. Following co-pretreatment with or without IGF-1 and linsitinib, MDA-MB-453 cells were exposed to lapatinib for 1h (**J**) and tucatinib for 36h (**K**). Transcript levels of NRF2 targets were measured using RT-qPCR with specific primers and normalized against actin. **L & M**. Measurement of nuclear localization of NRF2 after lapatinib and tucatinib treatment. Following the co-pretreatment strategy, MDA-MB-453 cells were exposed to lapatinib for 1h (**L**) and tucatinib for 36h (**M**). Immunofluorescence assays were performed to detect the nuclear translocation of NRF2 (red), counterstained with DAPI (blue). Images were captured at 100X using a confocal microscope. **N & O**. Evaluation of nuclear localization of NRF2 in a time-dependent manner post-lapatinib and tucatinib treatment. MDA-MB-453 cells were treated with 3.5 μ M lapatinib for 1, 3, 6, 10, and 24h (**N**) and 5 μ M tucatinib for 12, 24, 36, 40 and 48h (**O**) followed by an immunofluorescence assay to detect the nuclear localization of NRF2 (red), counterstained with DAPI (blue). **P & Q**. Evaluation of correlation between mitochondrial ROS production, IGF-1, IGF-1R and nuclear NRF2 expression by HER2-positive breast cancer cells after lapatinib and tucatinib treatment. As mentioned in Fig. 4J-K, along with mitochondrial ROS, IGF-1 and IGF-1R expression, nuclear NRF2 expression was also measured at the same time points following lapatinib (**P**) and tucatinib (**Q**) treatment. **R**. Correlation analysis between NRF2, IGF-1, IGF-1R and mitochondrial ROS post-lapatinib and tucatinib treatment using Python code. **S & T**. Measurement of pFOXO1/3a protein expression post-lapatinib and tucatinib exposure. As described, whole-cell lysates were harvested, lysed, and subjected to immunoblot analysis with pFOXO1/3a-specific antibody after lapatinib (**S**) and tucatinib (**T**) exposure. GAPDH was used as a loading control.

Experiments were repeated up to three times with biological replicates. Data points represent the average of three replicates with standard error (SE). The p-values among different comparisons are included in the Supplemental Table 7.

Conditioned media from IGF-1-pretreated tumor cells suppress oxidative stress in macrophages and promote M2-like polarization.

Cellular adaptation to targeted therapy not only promotes tumor cell survival but also alters stress responses and secretory programs, thereby reshaping the TME. Elevated oxidative stress promotes inflammation and anti-tumor M1 macrophage polarization³⁸. HER2-targeted TKIs such as lapatinib can

trigger stress-associated signals that suppress pro-tumorigenic M2 polarization^{39,40}. Because extratumoral IGF-1 suppresses TKI-induced oxidative stress via the PI3K/FOXO/NRF2 pathway, and IGF-1R/NRF2 signaling links to M2-like macrophage polarization^{41,42}, we hypothesized that IGF-1-mediated redox adaptation modifies the tumor secretome, suppressing inflammatory M1 macrophages and promoting an immunosuppressive M2 phenotype.

RAW264.7 macrophages were treated with M1 (LPS + IFN- γ) or M2 (IL-4) stimuli for 24h, then analyzed for expression of iNOS and ARG-1 as M1 and M2 markers (Supplemental Figure S8). To examine if tumor-secreted factors affect macrophages, RAW264.7 cells were exposed for 24h to serum-free conditioned media (CM) from MDA-MB-453 cells treated as in Fig. 5A. Live-cell imaging showed increased mitochondrial ROS in macrophages exposed to CM from lapatinib- (Fig. 5B) or tucatinib-treated (Fig. 5C) tumor cells. CM from IGF-1-pretreated cells significantly reduced ROS production and shifted macrophage phenotype: cells exposed to CM from TKI-treated tumor cells showed upregulation of the M1 marker iNOS, whereas CM from IGF-1-pretreated tumor cells suppressed iNOS and increased the M2 marker ARG-1 (Figs. 5D-E). Transcript analyses also showed increased levels of proinflammatory TNF- α in macrophages exposed to CM from lapatinib- and tucatinib-treated tumor cells, whereas CM from IGF-1-pretreated cells increased expression of the immunosuppressive cytokines TGF- β (both TKIs) and IL-10 (tucatinib only) (Supplemental Figure S9).

IGF-1-mediated secretome remodeling promotes resistance to macrophage-mediated immune clearance.

We next examined whether IGF-1-induced secretome remodeling influences tumor susceptibility to macrophage-mediated immune clearance. CM collected from MDA-MB-453 cells treated as shown in Fig. 5F was applied to drug-naïve CellTracker Orange-labeled MDA-MB-453 cells for 24h. RAW264.7 macrophages were independently activated to an M1 phenotype with LPS and IFN- γ , labeled with CellTracker Green, and co-cultured with tumor cells at a 1:1 ratio for 48h. MDA-MB-453 cells exposed to CM derived from IGF-1-pretreated and TKI-treated cells exhibited significantly increased resistance to macrophage-mediated clearance compared with cells exposed to CM from TKI-treated cells alone (Figs. 5G-H).

PI3K/NRF2 signaling is required for IGF-1-mediated immune evasion.

To determine if PI3K and NRF2 mediate IGF-1-dependent macrophage modulation, we inhibited IGF-1R, PI3K, and NRF2 with linsitinib, BEZ-235, and CT before CM collection, as shown in Fig. 5A. Blocking these pathways restored mitochondrial ROS in IGF-1-suppressed RAW264.7 macrophages (Figs. 5I-J). It also reversed IGF-1's protective effect against macrophage-mediated clearance (Figs. 5K-L).

Although CM experiments demonstrate the paracrine effects of tumor-secreted factors, the tumor microenvironment (TME) also involves continuous, direct contact between tumor and immune cells. To model this more directly, we developed direct co-culture models in which we treated MDA-MB-453 cells with lapatinib or tucatinib, with or without IGF-1 and inhibitors (linsitinib, BEZ-235, or CT), before co-

culturing them with LPS + IFN- γ -activated RAW264.7 macrophages. IGF-1-pretreated tumor cells escaped macrophage-mediated clearance, but inhibiting the PI3K/NRF2 pathway abolished this protection, restoring macrophage-mediated cytotoxicity to baseline levels (Supplemental Figure S10). We reproduced these results with THP-1-derived macrophages (Supplemental Figure S11).

These findings identify the PI3K/NRF2 pathway as critical for IGF-1-driven immune evasion. Together, they demonstrate that IGF-1-mediated redox adaptation helps HER2-positive tumor cells evade macrophage-mediated clearance by modulating macrophage polarization and function.

Figure 5. Lapatinib and tucatinib promote macrophage polarization to the M1 phenotype, but this shift is reversed to M2 by IGF-1/PI3K/NRF2 signaling. **A.** Experimental design for assessing the impact of lapatinib and tucatinib-treated (with or without IGF-1) cancer cells on RAW264.7 macrophage polarization. MDA-MB-453 cells were divided into seven groups: two untreated and five pretreated with 50ng/ml IGF-1 alone or in combination with 100nM BEZ-235, 250nM camptothecin (CT), or 40 μ M linsitinib for 6h. Cells were then exposed to 3.5 μ M lapatinib or 5 μ M tucatinib for 24h, incubated in serum-free medium for 24h, and then collected conditioned medium (CM). Drug naïve RAW264.7 macrophages were then exposed to the CM for 24h before performing various assays. **B & C.** Assessment of mitochondrial ROS production in macrophages. Following 24h treatment with CM from lapatinib (**B**) and tucatinib (**C**)-treated MDA-MB-453 cells, mitochondrial ROS formation was assessed in RAW264.7 cells using MitoSox dye. **D & E.** Measurement of iNOS and ARG-1 protein levels in macrophages. Following 24h treatment with CM from lapatinib (**D**) and tucatinib (**E**)-treated MDA-MB-453 cells, RAW264.7 cells were lysed and subjected to immunoblot analyses for iNOS (M1-specific marker) and ARG-1 (M2-specific marker). GAPDH was used as a loading control. **F.** Experimental design for assessing the impact of lapatinib and tucatinib-treated (with or without IGF-1) cancer cells on macrophage-mediated immune clearance. MDA-MB-453 cells were divided into seven groups: two untreated and five pretreated with IGF-1 alone or in combination with BEZ-235, camptothecin (CT), or linsitinib for 6h. Following pretreatment, cells were exposed to 3.5 μ M lapatinib or 5 μ M tucatinib for 24h, then incubated in serum-free medium for 24h to collect conditioned medium (CM). Drug-naïve MDA-MB-453 cells labeled with CellTracker Orange were treated with the CM collected for 24h and co-cultured with RAW264.7 macrophages activated with LPS and IFN- γ , labeled with CellTracker Green, at a 1:1 ratio for 48h before performing immunofluorescence assays. **G & H.** Measurement of MDA-MB-453 cell number post-lapatinib CM (**G**) and tucatinib CM (**H**) treatment after co-culturing with activated macrophages for immune clearance. **Using** this strategy, we counted the average number of MDA-MB-453 cells. **I & J.** Assessment of mitochondrial ROS production by RAW264.7 macrophages after pharmacological blockage of PI3K, NRF2 and IGF-1R. After treating the macrophages with CM from lapatinib (**I**) and tucatinib (**J**) plus/minus the above-mentioned inhibitor-treated MDA-MB-453 cells (as outlined in A), mitochondrial ROS production was assessed using the MitoSox dye. **K & L.** Measurement of RAW264.7 macrophage-mediated immune clearance of MDA-MB-453 cells exposed to CM from lapatinib (**K**) and tucatinib (**L**) plus/minus PI3K, NRF2, and IGF-1R inhibitor-treated cells (as mentioned in F), the average number of MDA-MB-453 cells was counted.

Experiments were repeated up to three times with biological replicates. Data points represent the average of three replicates with standard error (SE). The p-values among different comparisons are included in Supplemental Table 8.

IGF-1 suppresses oxidative stress during macrophage M1 activation.

Beyond paracrine signaling within tumor cells, IGF-1 directly promotes M2 macrophage polarization across multiple tissues by acting through the IGF-1R/PI3K/AKT pathway in macrophages themselves.^{43,44} NRF2 is also a key regulator of M2 macrophage programming, promoting key genes such as HO-1 and ARG-1⁴⁵. Based on our findings that extratumoral IGF-1 protects TKI-treated HER2-positive breast cancer cells via PI3K/FOXO/NRF2 signaling, we hypothesized that IGF-1 directly influences macrophage polarization through the same PI3K/NRF2 axis.

We pretreated RAW264.7 macrophages with IGF-1 (50ng/ml), alone or with linsitinib (40μM), for 6h before M1 activation with LPS and IFN-γ (Fig. 6A). M1 activation increased mitochondrial ROS³⁸, but IGF-1 reduced it. Linsitinib reversed IGF-1's effect, confirming IGF-1/IGF-1R signaling counteracts M1-related oxidative stress (Fig. 6B). We validated these findings in human THP-1 monocytes differentiated to macrophages by PMA and subsequently activated with LPS/IFN-γ. Consistent with RAW264.7 results, IGF-1 pretreatment suppressed mitochondrial ROS, while linsitinib restored oxidative stress levels (Supplemental Figure S12).

IGF-1-mediated macrophage modulation protects tumor cells from immune clearance.

After establishing that IGF-1 modulates macrophage oxidative stress, we examined its effect on tumor cell clearance. RAW264.7 macrophages were pretreated with IGF-1, with or without IGF-1R, PI3K, and NRF2 inhibitors, then co-cultured with CellTracker Orange-labeled MDA-MB-453 cells for 48h (Fig. 6C). Imaging and quantification showed IGF-1 pretreatment protected tumor cells from immune clearance. Inhibiting IGF-1R, PI3K, or NRF2 reversed this effect, restoring macrophage-mediated tumor cell elimination (Fig. 6D). These results were also reproduced in THP-1 macrophages (Supplemental Figure S13).

These findings confirm that the IGF-1/PI3K/NRF2 axis acts directly within macrophages, independent of tumor-derived signals, to suppress macrophage-mediated anti-tumor immunity.

IGF-1 protects tumor organoids through PI3K/NRF2-dependent immune modulation.

To mimic physiological conditions, we grew MDA-MB-453 cells as 3D tumor organoids and pretreated them with IGF-1 alone or with inhibitors of PI3K, NRF2, or IGF-1R. These organoids, labeled with CellTracker Orange, were co-cultured for 48h with activated CellTracker Green macrophages. Live-cell imaging showed that IGF-1-treated organoids retained more structural integrity, indicating protection from macrophages. Inhibition of PI3K, NRF2, or IGF-1R abolished this protection and disrupted organoids, even in the presence of IGF-1 (Fig. 6E). Collectively, these findings demonstrate that

extratumoral IGF-1 suppresses macrophage-mediated anti-tumor immunity by activating the PI3K/NRF2 signaling axis in both macrophages and HER2-positive breast cancer cells.

Figure 6. IGF-1 directly alters macrophage polarization and protects against the clearance of cancer cells in a PI3K/NRF2-dependent manner. **A.** Experimental design for the assessment of ROS generation post-macrophage activation. RAW264.7 macrophages were divided into three groups: one untreated and two pretreated with 50ng/ml IGF-1 alone or with 40μM linsitinib, followed by activation with LPS and IFN-γ. These cells were then subjected to an immunofluorescence assay. **B.** Assessment of mitochondrial ROS. Following the strategy mentioned in A, mitochondrial ROS in RAW264.7 macrophages with IGF-1 plus/minus linsitinib treatment was measured using the MitoSox dye. **C.** Experimental design for assessing RAW264.7 macrophage-mediated clearance of cancer cells. RAW264.7 macrophages were divided into five groups; one untreated and four treated with IGF-1 alone or in combination with IGF-1R/PI3K/ NRF2 inhibitors for 6h. Cells were then activated with LPS and IFN-γ for 24h and tracked with CellTracker Green. Drug naïve MDA-MB-453 cells were labeled with cell tracker orange and co-cultured with activated RAW264.7 macrophages in a 1:1 ratio for 48h, after which immunofluorescence assay was performed. **D.** Assessment of clearance of cancer cells co-cultured with IGF-1-treated macrophages with or without treatment with inhibitors of the IGF-1R/PI3K/NRF2 axis. As mentioned in the strategy in C, the average number of CellTracker Orange-labeled MDA-MB-453 cells was counted after co-culturing with activated macrophages (treated with IGF-1 and IGF-1R/PI3K/NRF2 inhibitors) to check for immune clearance. **E.** Assessment of IGF-1 plus/minus IGF-1R/PI3K/NRF2 inhibitor-treated MDA-MB-453 organoid clearance from activated RAW264.7 macrophages.

The p-values among different comparisons are included in Supplemental Table 9.

Clinical association of the IGF-1/PI3K/NRF2 axis with M2 macrophage infiltration in human breast cancer.

After demonstrating the dual tumor-protective and immune-modulatory roles of the IGF-1/PI3K/NRF2 pathway in vitro, we examined its association with macrophage polarization in human breast cancer. Using TIMER2.0, we computed purity-adjusted partial Spearman correlations between key pathway elements (IGF1, PIK3CA, and NRF2/NFE2L2) and canonical M2 macrophage markers (CD163, MRC1/CD206 [13,14], ARG1, TGFB1, and IL10) in the pooled TCGA-BRCA cohort and in the BRCA-Her2 and BRCA-LumB molecular subtypes (Supplemental Tables S10, S11 and S12).

In the pooled BRCA cohort, IGF1 expression showed significant positive correlations with all five M2 markers, strongest with MRC1 ($\rho = 0.309$, $p = 2.09 \times 10^{-23}$) and TGFB1 ($\rho = 0.297$, $p = 9.84 \times 10^{-22}$), and more modest with IL10 ($\rho = 0.115$, $p = 2.72 \times 10^{-4}$), ARG1 ($\rho = 0.111$, $p = 4.67 \times 10^{-4}$), and CD163 ($\rho = 0.075$, $p = 1.80 \times 10^{-2}$) (Fig. 7A).

PIK3CA, which encodes the catalytic subunit of PI3K, showed a similar pattern and correlated significantly with MRC1 ($\rho = 0.401$, $p = 8.71 \times 10^{-40}$), CD163 ($\rho = 0.388$, $p = 4.69 \times 10^{-37}$), IL10 ($\rho = 0.313$, $p = 4.75 \times 10^{-24}$), and ARG1 ($\rho = 0.119$, $p = 1.70 \times 10^{-4}$), but not with TGFB1 ($\rho = 0.021$, $p = 0.516$) (Fig. 7A).

NFE2L2 (NRF2) correlated significantly with all five markers: MRC1 ($\rho = 0.280$, $p = 2.34 \times 10^{-19}$), IL10 ($\rho = 0.253$, $p = 5.77 \times 10^{-16}$), CD163 ($\rho = 0.230$, $p = 1.99 \times 10^{-13}$), TGFB1 ($\rho = 0.114$, $p = 3.22 \times 10^{-4}$), and ARG1 ($\rho = 0.084$, $p = 7.97 \times 10^{-3}$) (Fig. 7A).

These associations were largely preserved, though less comprehensively, in the BRCA-Her2 and BRCA-LumB subtypes, likely reflecting their smaller sample sizes ($n = 82$ and $n = 219$, respectively, versus $n = 1,100$ pooled). MRC1 remained significantly correlated with IGF-1, PIK3CA, and NRF2 in both subtypes, as did CD163, except for NFE2L2 in BRCA-LumB ($p = 0.055$). ARG1 and TGFB1 correlations were largely attenuated to non-significance in both subtypes, while IL10 remained significant for IGF1 in BRCA-Her2 and for NFE2L2 in BRCA-LumB (Fig. 7A).

To further resolve the cellular source of this signal, we examined single-cell RNA-seq data from previously used breast cancer datasets (BRCA_GSE114727_inDrop and BRCA_GSE138536) using TISCH2 (Figs. 7B-C). IGF-1R was consistently expressed in Mono/Macro populations in both datasets, supporting direct macrophage responsiveness to IGF-1. IGF-1 itself was predominantly expressed by fibroblasts and myofibroblasts, consistent with the stromal source established earlier (Fig. 1C-G). Among the M2 markers, CD163 showed macrophage-associated expression in BRCA_GSE114727_inDrop, whereas both CD163 and MRC1 showed broader expression across fibroblasts and other stromal populations in BRCA_GSE138536, a caveat to consider when interpreting these markers as M2-specific readouts in the bulk correlation analysis above.

These findings support a clinical association between IGF-1/PI3K/NRF2 pathway activity and an M2 macrophage-rich, immunosuppressive tumor microenvironment in breast cancer. The association is most robust in the pooled cohort and consistent across molecular subtypes for the canonical M2 markers CD163 and MRC1.

Figure 7: Clinical correlation between the IGF-1/PI3K/NRF2 pathway and M2 macrophage infiltration with single-cell validation of pathway component expression. **A.** Correlation between IGF-1, PIK3CA (encoding the PI3K catalytic subunit), and NFE2L2 (NRF2) expression and canonical M2 macrophage markers (CD163, MRC1/CD206, ARG1, TGFB1, IL10) in the TCGA breast invasive carcinoma (BRCA) cohort, analyzed using TIMER2.0. Correlations were computed as purity-adjusted partial Spearman's correlations for the pooled BRCA cohort and the BRCA-Her2 and BRCA-LumB molecular subtypes. Dot color represents Spearman's ρ (red, positive; blue, negative); dot size represents statistical significance (larger dots indicate smaller p-values, as shown in the size legend). Faded/open dots indicate non-significant correlations ($p \geq 0.05$). **B-C.** Single-cell RNA-seq expression of CD163, IGF-1, IGF-1R, and MRC1 (**B**) or CD163, IGF-1, and IGF-1R (**C**) across major cell types in two independent human breast cancer datasets, BRCA_GSE114727_inDrop (**B**) and BRCA_GSE138536 (**C**), visualized using TISCH2. Violin plots show the expression distribution of each gene within each annotated cell type.

Discussion

The rapid emergence of adaptive resistance (RD) limits the use of HER2-targeted TKIs lapatinib and tucatinib. Transcriptomic analysis indicated that RD involves activation of IGF-1/PI3K/AKT signaling in a growth-arrested, non-proliferative state, alongside suppression of proliferative and mTORC1 signaling (Fig. 1). ROS-dependent DNA damage further contributed to this stress phenotype, as evidenced by increased 53BP1 foci following TKI treatment (Fig. 2). We discovered a non-canonical resistance mechanism where therapy-induced oxidative stress makes tumors rely on stromal IGF-1 signaling. This extends prior work showing that HER2 downregulation itself triggers oxidative stress and compensatory NRF2 activation to drive dormant tumor cell recurrence ⁴⁶, by identifying stromal IGF-1 as a non-cell-autonomous, upstream trigger of this same NRF2-dependent survival program. Consistent with this, lapatinib RD tumors showed significant upregulation of SLC7A11, GCLC, and GPX4 (key genes in glutathione-dependent redox defense), whereas canonical NRF2 targets such as NQO1 and HMOX1 were not induced at this time point (Supplemental Table S1), potentially reflecting the transient kinetics of NRF2 activation observed in vitro (Fig. 4). By day 14, a subset of target genes may already have returned toward baseline in RD tumors. The selective upregulation of SLC7A11 and GPX4, key regulators of ferroptosis defense, suggests that IGF-1/NRF2-adapted persister cells become dependent on this specific redox axis, potentially creating a ferroptosis vulnerability analogous to that described in drug-tolerant persister cells more broadly ⁴⁷. Single-cell transcriptomics identified the tumor stroma as the primary source of IGF-1 (Fig. 1), and functional studies demonstrated that exogenous IGF-1 reverses TKI-induced oxidative stress and cell death (Figs. 2–3).

Fibroblasts like MEFs are known sources of IGF-family growth factors supporting neighboring cell survival through paracrine signaling (43). Although they are not definitive cancer-associated fibroblasts, we used these cells as a proxy for stromal IGF-1 secretion, co-cultured them with MDA-MB-453 cells, used an IGF-1-neutralizing antibody, and added back recombinant IGF-1 at 3x excess. We found that bioactive IGF-1 secreted by MEFs is important for protecting against lapatinib-induced oxidative stress (Supplemental Figure S14). Neutralizing IGF-1 partially reversed MEF-mediated protection against ROS and cell death, indicating IGF-1 is a key but not the only protective factor (Supplemental Figure S14). We further knocked down IGF-1R in MDA-MB-453 cells before co-culturing with MEFs and measured ROS and cell viability. As expected, unlike control siRNA-transfected cells, IGF-1R-depleted cells could not reverse lapatinib-induced ROS and cytotoxicity (Supplemental Figure S15), confirming the role of IGF-1 signaling in MEF-mediated protection from TKI-induced oxidative stress in MDA-MB-453 cells.

This IGF-1 rescue of TKI-induced oxidative stress via the PI3K/NRF2 axis was also observed in HCC1954, another HER2-positive breast cancer cell line, suggesting it is broader than MDA-MB-453 cells (Supplemental Figure S16). These findings support a model in which HER2-positive tumors leverage stromal IGF-1-driven tissue repair mechanisms to create an antioxidant niche that helps survival under therapeutic stress.

The link between TKI-induced mitochondrial ROS and activation of the IGF-1R/NRF2 axis (Fig. 4) highlights oxidative stress as more than a toxic byproduct of HER2 inhibition; it acts as a key signal that enables persister cell adaptation. By increasing NRF2 and IGF-1R, tumor cells quickly develop the

signaling needed to use stromal IGF-1 and restore redox balance. Our findings further demonstrate that transient stromal rescue yields durable phenotypic consequences. Rechallenge experiments revealed that IGF-1-mediated survival establishes a persistent NRF2-dependent antioxidant state, with lower basal ROS levels and increased tolerance to subsequent oxidative stress. This adaptation conferred cross-resistance to mechanistically distinct ROS-inducing therapies, including doxorubicin and tucatinib (Fig. 3). However, this persistent antioxidant adaptation also created collateral vulnerability to broad pan-ErbB inhibition. High-dose lapatinib rechallenge triggered extensive ROS accumulation and cell death in adapted persister populations, suggesting that sustained antioxidant defense becomes critically dependent on compensatory ErbB signaling networks. Consistent with previous reports of EGFR and HER4 compensation during HER2-targeted therapy resistance^{48,49}, our findings support a model in which persister cells increasingly rely on alternative ErbB signaling to maintain PI3K/AKT-dependent NRF2 stabilization and redox homeostasis.

Our data link extracellular stromal signals to intracellular antioxidant responses. While canonical models interpret PI3K/AKT activation during HER2 TKI resistance primarily as a proliferative and anti-apoptotic response^{50,51}, our findings indicate that therapy-induced oxidative stress reprograms this pathway to maintain redox balance. We propose that stromal IGF-1 initially activates PI3K/AKT signaling, suppresses FOXO activity, and stabilizes NRF2 by disrupting KEAP1-mediated degradation. As persister cells adapt, compensatory EGFR and HER4 signaling sustain this antioxidant circuitry by maintaining continuous PI3K/AKT activity. Selective HER2 inhibition with tucatinib spares these pathways, but broad pan-ErbB inhibition with high-dose lapatinib disrupts this signaling, destabilizing NRF2, causing redox imbalance, and cell death. These findings link stromal signaling, redox regulation, and TKI resistance.

We found that IGF-1-mediated TKI resistance also has an immunological component: activation of the IGF-1/PI3K/NRF2 axis changes the tumor secretome and drives macrophages to polarize into a pro-tumor M2 type, reducing tumor clearance (Figs. 5–6). We reproduced these findings in tumor organoid-macrophage co-cultures using IGF-1R, PI3K, and NRF2 inhibitors (Fig. 6). Bioinformatics analyses indicated positive correlations between IGF-1/PI3K/NRF2 pathway activity and M2 macrophage infiltration in human breast tumors (Fig. 7).

In summary, we identified stromal IGF-1-driven redox adaptation as a key mechanism behind HER2 TKI persistence and residual disease. This finding supports combining HER2-TKIs with IGF-1R/PI3K/NRF2 pathway inhibitors to target antioxidant defense and M2 macrophage-mediated immune suppression. Since IGF-1 and NRF2 protect normal tissues from oxidative damage, careful consideration of therapy timing and toxicity will be essential. We confirmed this by replicating IGF-1-mediated protection against TKI-induced oxidative stress in non-cancerous cells (Supplemental Figure S17), implying risks of side effects with systemic pathway inhibition. While our study uses clinical data, future *in vivo* work is needed to explore macrophage-stroma-tumor interactions during resistance.

Conclusion

This study redefines IGF-1's role in therapy resistance, showing that it shifts from an autocrine growth signal to a paracrine factor that maintains redox balance. HER2-targeted TKIs inhibit breast cancer cell growth but induce oxidative stress, forcing early persister cells to depend on the tumor microenvironment. IGF-1 from the stroma modulates PI3K/FOXO to stabilize NRF2, thereby neutralizing oxidative stress. This antioxidant response promotes tumor survival and a long-term redox-adapted state that enables phenotypic tolerance to oxidative stress. IGF-1-driven adaptation also reprograms the tumor secretome, promoting immunosuppressive M2 macrophage polarization and immune evasion. Our findings link stromal support to redox and immune adaptation and suggest that co-targeting IGF-1R/NRF2 with HER2 TKIs may eliminate residual disease and prevent resistance.

Methods

Cell lines, inhibitors, and Reagents:

Authenticated and mycoplasma-tested HER2-positive breast cancer MDA-MB-453, human hepatoma HepG2, mouse embryonic fibroblast MEF, human monocyte THP1, and rodent macrophage RAW264.7 cell lines were obtained from the National Center for Cell Science (Maharashtra, India) repository. Human mammary epithelial cells MCF10A and HER2-positive breast cancer cells HCC1954 were generously provided by Dr Carlos L. Arteaga (UT Southwestern Medical Center, Texas, USA). MCF10A cells were cultured as per published protocol ⁵², and all other cell lines were cultured as per the supplier's recommendation. We purchased routine chemicals/reagents from HiMedia Laboratories (Maharashtra, India) and Sigma-Aldrich (Missouri, USA). Supplemental Tables S3 and S4 provide the PCR primers (from Anuvanshiki OPC, Uttar Pradesh, India) and the antibodies, drugs, and inhibitors along with their sources.

Cell Proliferation, Viability and Rechallenge Assays:

Cell proliferation and viability were measured by manual cell counting, MTT assay, and calcein AM-PI live cell staining. Details are provided in the Supplemental Methods.

To assess long-term therapeutic memory, MDA-MB-453 persister cells that survived the initial 48h TKI exposure (with or without 50ng/ml IGF-1 pretreatment) were maintained for 15 days and subjected to TKI rechallenge, followed by ROS and viability measurements, as described in detail in the Supplemental Methods.

RNAi-mediated Gene Depletion and Real-time PCR:

Details of transfection of IGF-1R and NRF2 siRNAs in MDA-MB-453 cells, followed by RNA isolation and qRT-PCR, are provided in the Supplemental Methods. Relative mRNA levels were calculated by the previously published delta Ct method ³³.

Immunofluorescence, Live Cell Imaging and Immunoblot Assays:

These are described in detail in the Supplemental Methods.

Co-culture Assay:

MDA-MB-453 cells pre-treated with 50ng/ml IGF-1 for 6h in serum-free medium were labeled with 2 μ M CellTracker Orange CMRA dye (Thermo Fisher Scientific) for 2h. RAW264.7 macrophages were activated with 1 μ g/ml LPS and 35ng IFN- γ for 24h and labeled with 4 μ M CellTracker Green CMFDA dye for 2h. Labeled cells were mixed in a 1:1 ratio and imaged after 48h with a Nikon Eclipse Ti-2 confocal microscope, and quantified via ImageJ (National Institutes of Health, Maryland, USA).

For M2 polarization of RAW264.7 cells, the canonical stimulus IL-4 was used at 10ng/ml for 24h.

To determine the functional contribution of fibroblast-derived IGF-1, MDA-MB-453 cells were labeled with CellTracker Green CMFDA, then mixed with unlabeled MEF (1:1), as indicated, and pretreated with 5 μ g/ml IGF-1-neutralizing antibody (MAB2912, R&D Systems, Minnesota, USA) alone or pre-mixed with recombinant IGF-1 (3X excess). Cultures were then treated to 3.5 μ M lapatinib and examined for ROS production after 1h and for cell viability (Hoechst-positivity) after 48h by live cell imaging. In one experiment, before co-culturing, MDA-MB-453 cells were transfected with either a negative control (NT) or IGF-1R siRNA (25nM), as described in the Supplemental Methods.

Organoid (3D) Co-culture Assay:

MDA-MB-453 cells (2000 cells/ml) were mixed with growth factor-reduced Matrigel (Sigma-Aldrich) in a 1:1 ratio in complete media. Organoids were grown for 10 days, then incubated with 50ng/ml IGF-1 for 6h before co-culturing with activated macrophages. RAW264.7 macrophages were activated and labeled with CellTracker Green CMFDA as per manufacturer's recommendation. MDA-MB-453 organoids were labeled with CellTracker Orange CMRA and co-cultured with RAW264.7 macrophages in 1:2 ratio for 48 h. Organoids were imaged using a Nikon Eclipse Ti-2 confocal microscope and quantified with ImageJ. We assessed organoid structural integrity as described in the Supplemental file.

Statistical Analysis:

We used GraphPad Prism V8.0.1.244 (Dotmatics, Massachusetts, USA; www.graphpad.com) for statistical analysis. Each bar graph shows the mean of three technical replicates with standard error (SE). We repeated each experiment two to three times with different biological replicates. Significance levels for experiments are in Supplemental Tables S5-S9. For correlation analysis, we used Python 3.6.

Computational and Bioinformatics Analysis:

We performed single-cell and bulk transcriptomic tumor-immune profiling using TISCH2 ⁴⁴, GEO2R, and the TIMER2.0 web server ⁴⁶, with various patient datasets as described in detail in the Supplemental

Methods. We performed pathway/gene-set enrichment using Enrichr ⁴⁵ and cross-validated it with a second enrichment tool, ShinyGO ⁴⁷. We prepared illustrations using BioRender (<https://www.biorender.com>).

Declarations

Declaration of Competing Interests:

All authors of this manuscript declare no financial or non-financial competing interests.

Author Contribution

ND: Investigation, data curation, formal analysis, funding acquisition, validation, visualization, writing-original draft, writing-review and editing; GM: Investigation, data curation, formal analysis, validation, visualization; AC: Supervision, conceptualization, resources, data curation, formal analysis, funding acquisition, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing.

Acknowledgement

We thank Shiv Nadar Institution of Eminence and the Core Imaging Facility, and acknowledge funding from the Council of Scientific & Industrial Research (37WS(0102)/2023-24/EMR-II/ASPIRE to AC) and the Department of Science and Technology (DST/INSPIRE Fellowship/2021/IF210484 to ND). Our gratitude is extended to Mr. Yahya Ashraf for assistance with organoid structural analysis and to Dr. Goutam Chowdhury for manuscript feedback. During the preparation of this work, we used Google Gemini 3 Pro, Claude, and Grammarly Pro to assist with drafting, editing, and improving readability. Afterward, we reviewed and edited the content and took full responsibility for the published article.

Data Availability:

Bulk and single-cell RNA-Seq data were obtained from Gene Expression Omnibus (GEO), TISCH2, and TCGA. The GSEA results are provided in the manuscript, with details of the analysis in the Supplemental Methods section. We have not reported any unpublished data in this manuscript.

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Figures

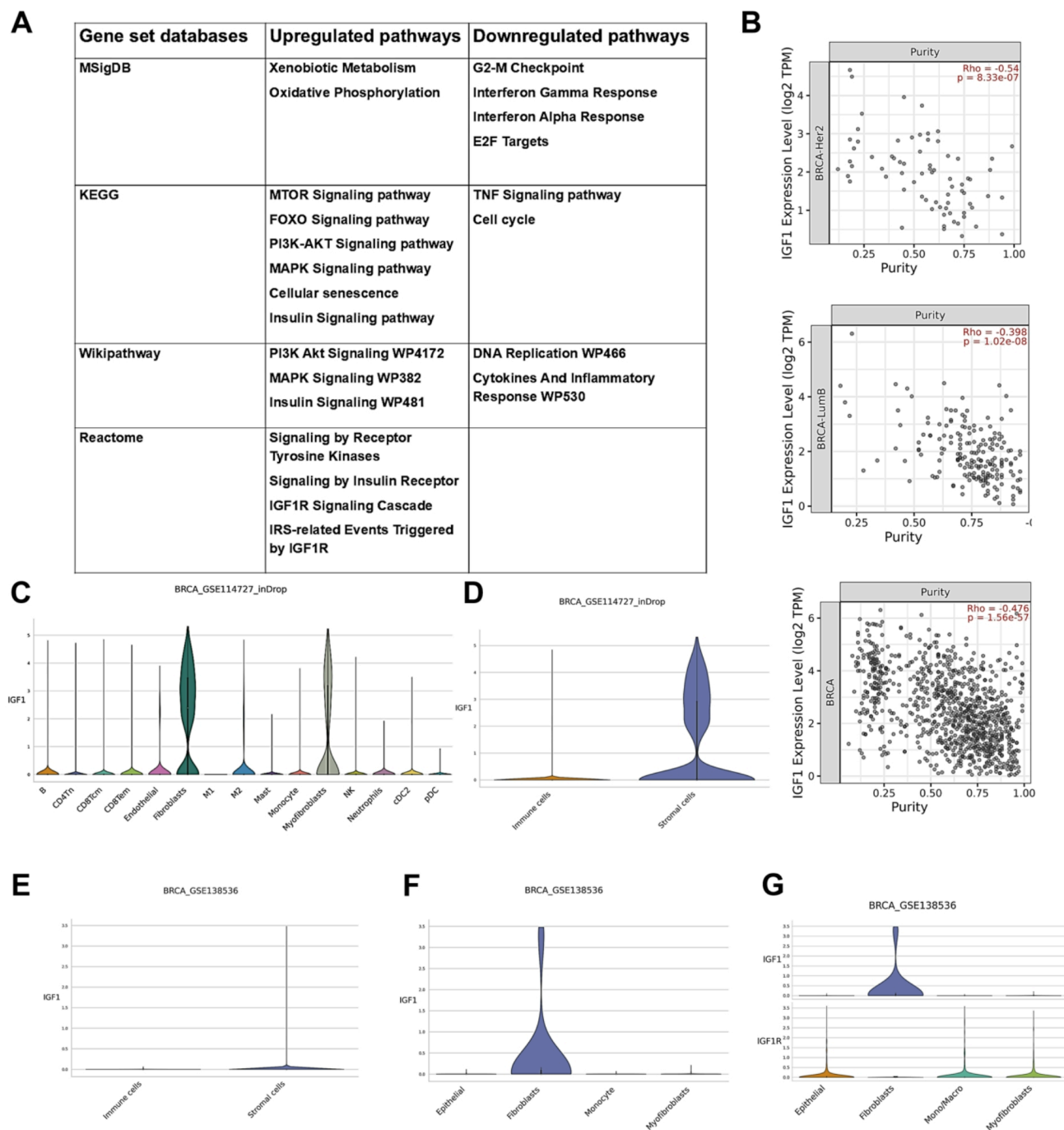


Figure 1

Residual disease exhibits a stress-adaptation signature, with fibroblasts identified as the primary source of IGF-1. **A.** Publicly available gene expression data (GSE130788) from HER2-positive breast cancer patients treated with lapatinib (residual disease/RD vs pre-treatment biopsies) were analyzed using Enrichr, an interactive gene enrichment analysis tool. Top pathways enriched in the RD, as per MSigDB, Reactome, Wikipathway, and KEGG, are shown. **B.** The TCGA-BRCA, BRCA-Her2, and BRCA-

LumB cohorts were queried via TIMER2.0 to assess the relationship between IGF-1 expression and tumor purity. **C-G**. The single-cell RNA sequencing datasets GSE114727_inDrop and GSE138536 from human breast cancer samples were queried via TISCH2 to compare IGF-1 expression densities across epithelial, stromal, and immune cell populations.

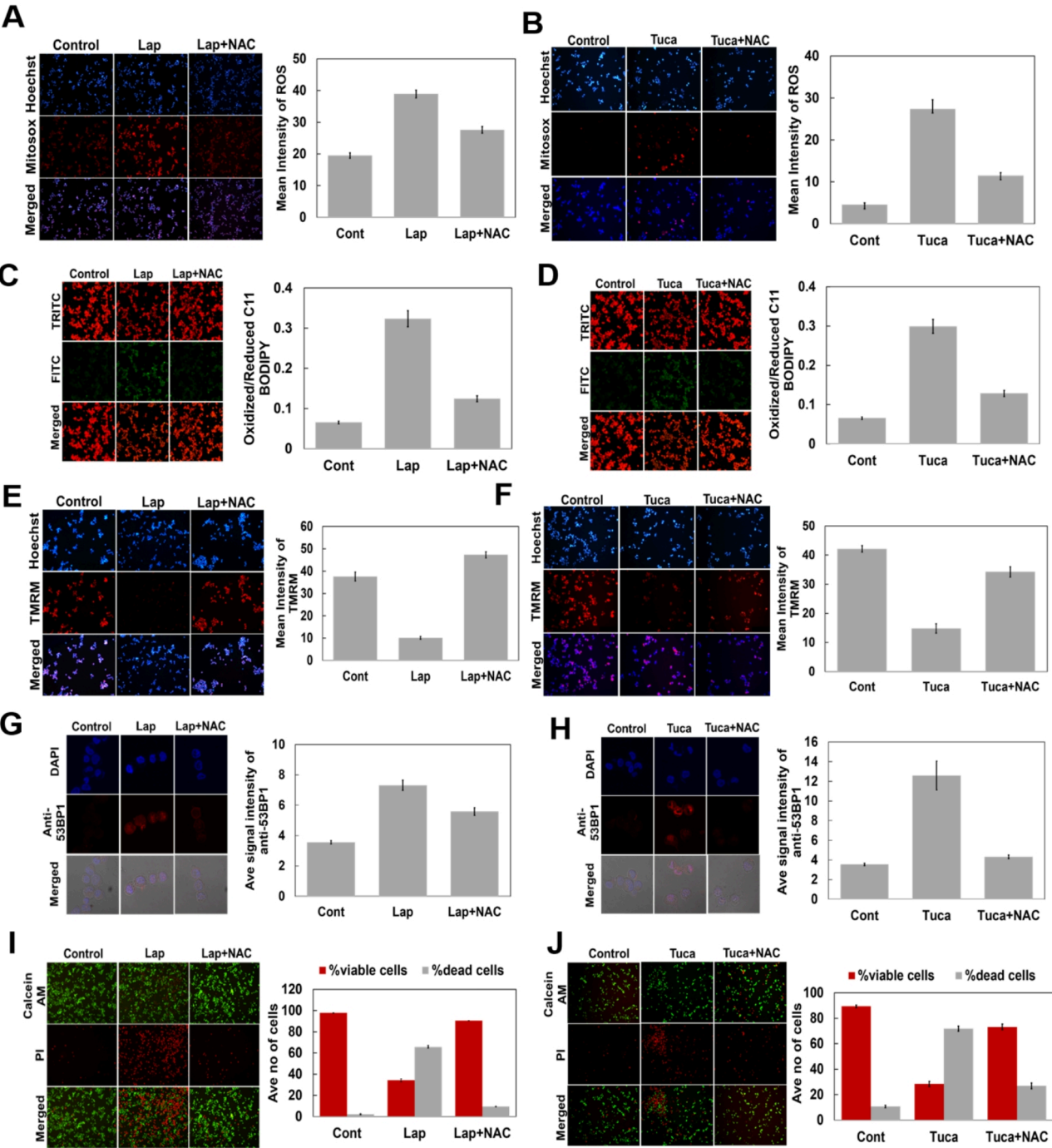


Figure 2

Lapatinib and tucatinib trigger reactive oxygen species production, DNA and mitochondrial damage and cytotoxicity in HER2-positive breast cancer cells, effects that N-Acetylcysteine (NAC) can counteract significantly. A & B. Assessment of mitochondrial ROS production. MDA-MB-453 cells were pretreated with or without 250µM NAC for 6h before exposing to 3.5mM lapatinib for 1h (**A**) and 5mM tucatinib for 36h (**B**). Live-cell images were captured at 20X magnification using an epifluorescence microscope. Blue channel represents Hoechst-stained nucleus and red represents MitoSOX, the mitochondrial ROS-indicator. Graphs show the quantification of mean signal intensity from multiple microscopic images. **C & D.** Assessment of lipid peroxidation. Lipid peroxidation was measured in MDA-MB-453 cells after 12h treatment with 3.5mM lapatinib (**C**) and 5mM tucatinib (**D**), pretreated with NAC as mentioned above. Images were captured at 20X (2X zoom) using a confocal microscope. Graphs represent the quantification of average signal intensity of oxidized (red) to reduced (green) C11-BODIPY staining. **E & F.** Assessment of mitochondrial membrane potential. Mitochondrial damage was assessed by measuring loss of mitochondrial membrane potential in lapatinib (**E**) and tucatinib (**F**)-treated MDA-MB-453 cells (as above) using TMRM (red), counterstained with Hoechst (blue). Live-cell images were captured at 20X using an epifluorescence microscope. **G & H.** Assessment of 53BP1 expression. Expression of the DNA damage repair protein 53BP1 was measured by immunofluorescence with an anti-53BP1 antibody (red) after lapatinib (**G**) and tucatinib (**H**) treatment with or without prior treatment with NAC as indicated above. Cells were counterstained with nuclear dye DAPI (blue), and images were captured at 100X with a confocal microscope. **I & J.** Assessment of cell viability and death. Live and dead cell numbers were measured after 48h of lapatinib (**I**) or tucatinib (**J**) treatment, with or without NAC pretreatment as mentioned above. Viable and dead cells were stained with calcein AM (green) and propidium iodide/PI (red), respectively. Images were captured at 20X in a confocal microscope. The bar graphs show the quantification of the average number of live and dead cells.

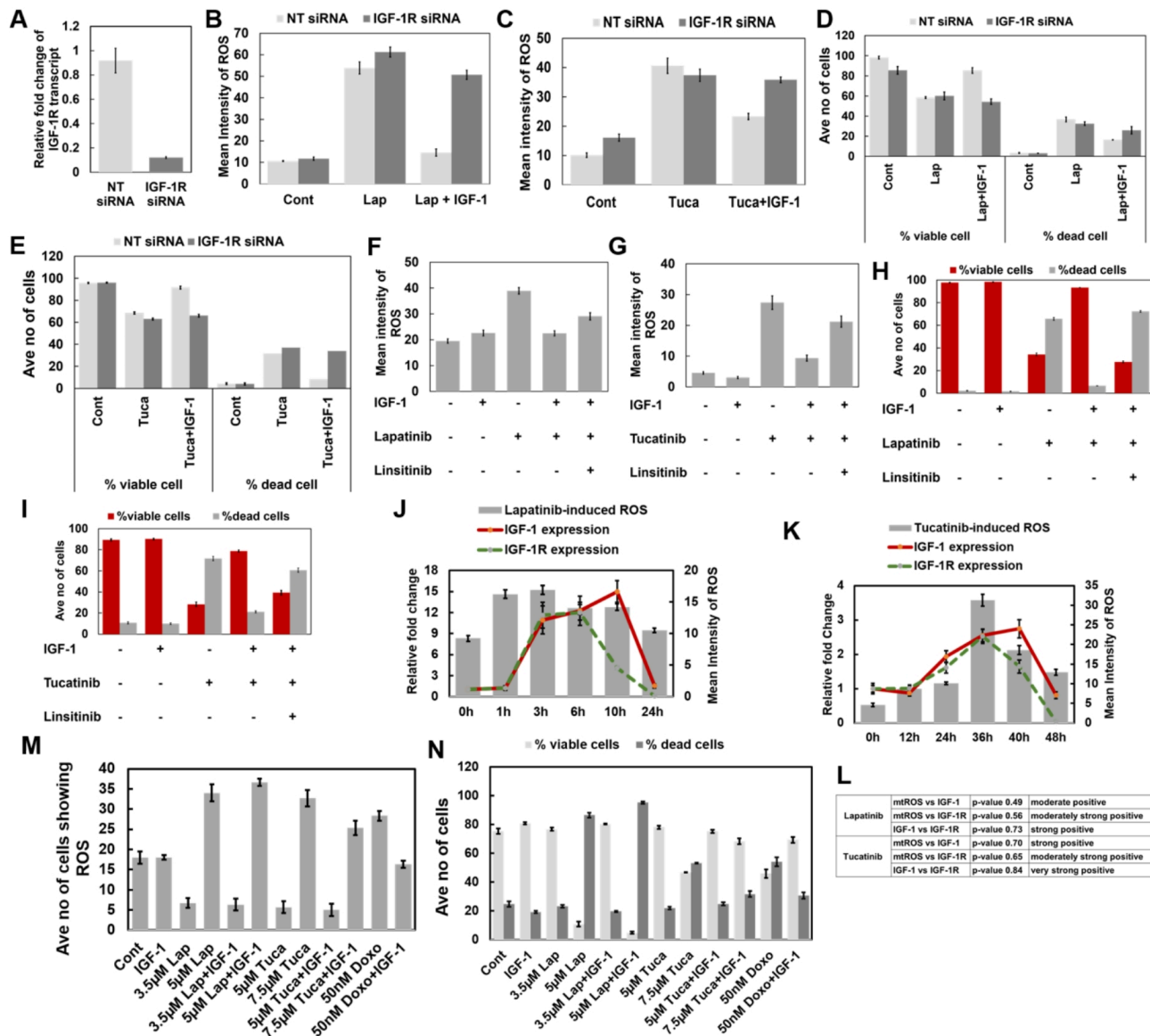


Figure 3

IGF-1 protects cells from lapatinib and tucatinib-induced ROS production and cytotoxicity through IGF1-R in HER2-positive breast cancer cells. **A.** Assessment of siRNA knockdown of IGF-1R. RT-qPCR confirms IGF-1R knockdown efficiency in MDA-MB-453 cells. **B & C.** Assessment of lapatinib and tucatinib-mediated mitochondrial ROS production after IGF-1R knockdown. MDA-MB-453 cells were pretreated with 50ng/ml IGF-1 for 6h before exposing them to 3.5mM lapatinib for 1h (**B**) and 5mM tucatinib for 36h (**C**). **D & E.** Assessment of lapatinib and tucatinib-mediated cytotoxicity after IGF-1R knockdown. Following 6h of pretreatment with IGF-1, MDA-MB-453 cells were exposed to 3.5mM lapatinib (**D**) and 5mM tucatinib (**E**) for 48h before measuring cytotoxicity with calcein-AM and PI dyes. The numbers of viable (calcein) and dead (PI) cells were counted and graphically represented. **F & G.** Assessment of lapatinib and tucatinib-mediated mitochondrial ROS production after pharmacological

inhibition of IGF-1R. MDA-MB-453 cells were pretreated with IGF-1R/INSR inhibitor linsitinib (40 μ M) and IGF-1 (50ng/ml) for 6h and then exposed to 3.5mM lapatinib for 1h **(F)** and 5mM tucatinib for 36h **(G)**. Mitochondrial ROS were measured via live-cell imaging with the MitoSOX dye, as previously described. **H & I.** Measurement of cytotoxicity after pharmacological inhibition of IGF-1 followed by lapatinib and tucatinib treatment. Following co-pretreatment, MDA-MB-453 cells were exposed to lapatinib **(H)** and tucatinib **(I)** for 48h. Viable and dead cells were measured via calcein-AM and PI staining. **J & K.** Evaluation of correlation between mitochondrial ROS production, IGF-1 and IGF-1R expression by HER2-positive breast cancer cells after lapatinib and tucatinib treatment. Mitochondrial ROS generation was measured in MDA-MB-453 cells after 3.5mM lapatinib **(J)** and 5mM **(K)** tucatinib treatment at different time points (lapatinib- 0, 1, 3, 6, 10, and 24h and tucatinib- 0, 12, 24, 36, 40, and 48h). Simultaneously, IGF-1 and IGF1-R expressions were measured at these time points via RT-qPCR, normalized against actin. **L.** Correlation between drug-induced mitochondrial ROS, IGF-1 and IGF-1R expressions using Python code. **M & N.** Exploring persistent eco-evolutionary memory against drug-induced oxidative stress following transient IGF-1-mediated rescue. MDA-MB-453 cells surviving the initial 48h TKI exposure (with or without 6h IGF-1 pretreatment) were isolated and cultured in standard drug-free conditions. Following a 15-day recovery, equal numbers of persister cells from both IGF-1-pre-treated and untreated TKI-treated groups were rechallenged in serum-free media with either lapatinib/tucatinib or doxorubicin. Mitochondrial ROS generation **(M)** and cytotoxicity **(N)** were measured to quantify the stable, broad-spectrum antioxidant capacity conferred by prior transient IGF-1 exposure.

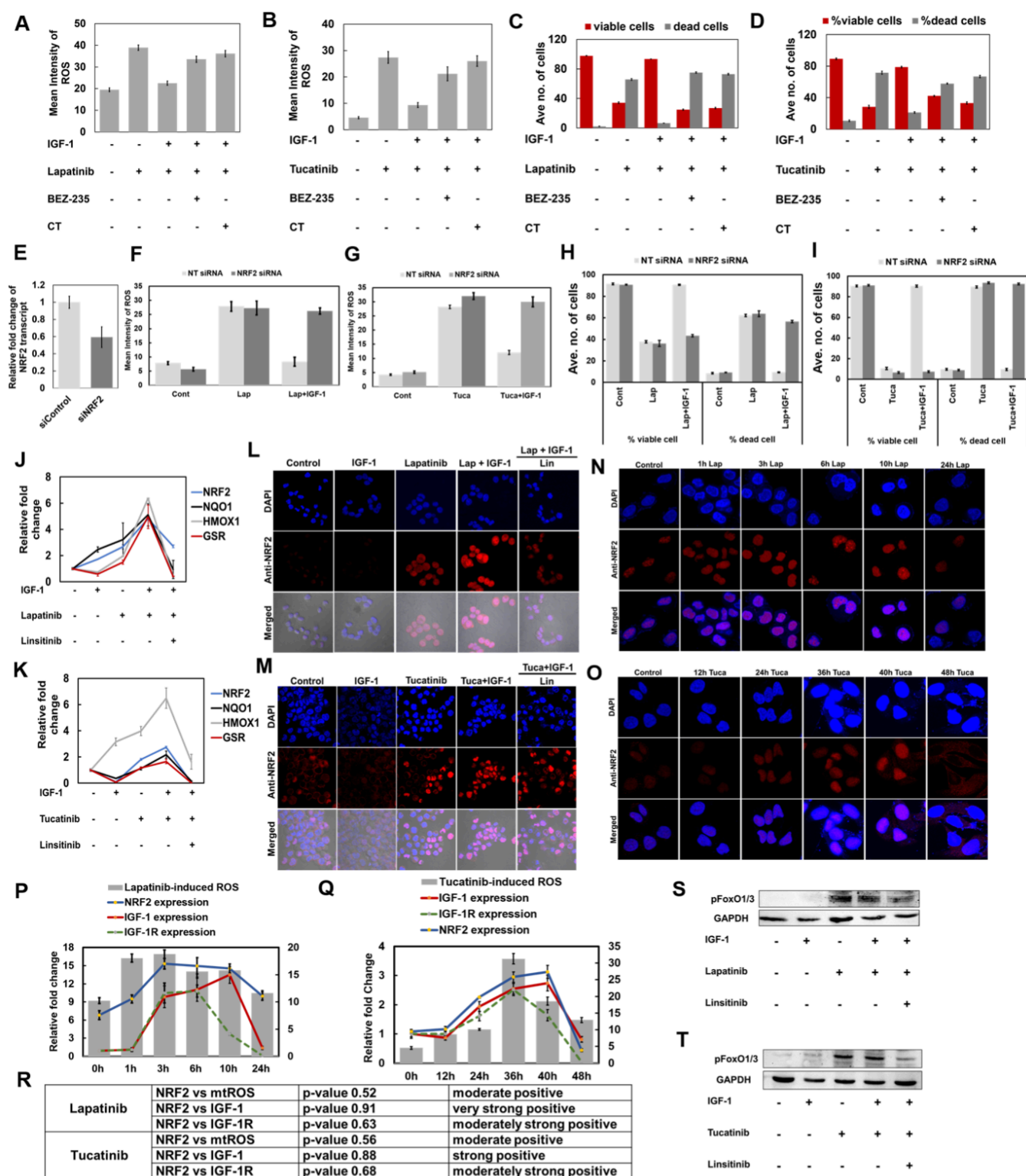


Figure 4

IGF-1 protects HER2-positive breast cancer cells from lapatinib and tucatinib-induced oxidative stress through the PI3K/NRF2 pathway. **A & B.** Assessment of lapatinib and tucatinib-induced ROS after pharmacological inhibition of PI3K and NRF2. MDA-MB-453 cells were pretreated with 50ng/ml IGF-1 with or without (100nM) BEZ-235-PI3K inhibitor and (250nM) Camptothecin (CT)-NRF2 inhibitor for 6h before exposing them to 3.5mM lapatinib for 1h (**A**) and 5mM tucatinib for 36h (**B**). **C & D.** Assessment of

lapatinib and tucatinib-induced cytotoxicity after pharmacological inhibition of PI3K and NRF2. Following pretreatment with IGF-1 with or without BEZ-235 and CT for 6h (as above), MDA-MB-453 cells were exposed to lapatinib **(C)** and tucatinib **(D)** for 48h to measure cytotoxicity with calcein-AM (live cell) and PI (dead cell) dyes. **E.** Assessment of siRNA-mediated knockdown of NRF2. RT-qPCR confirming NRF2 knockdown efficiency in MDA-MB-453 cells. **F & G.** Measurement of mitochondrial ROS after genetic ablation of NRF2 followed by lapatinib and tucatinib treatment. Following NRF2 RNAi, MDA-MB-453 cells were first exposed to IGF-1, followed by lapatinib **(F)** and tucatinib **(G)** treatment. **H & I.** Evaluation of lapatinib and tucatinib-mediated cytotoxicity following NRF2 knockdown. After pretreatment with IGF-1, MDA-MB-453 cells were exposed to **(H)** lapatinib and **(I)** tucatinib for 48h to measure cytotoxicity. **J & K.** Measurement of transcript levels of NRF2 targets after lapatinib and tucatinib treatment. Following co-pretreatment with or without IGF-1 and linsitinib, MDA-MB-453 cells were exposed to lapatinib for 1h **(J)** and tucatinib for 36h **(K)**. Transcript levels of NRF2 targets were measured using RT-qPCR with specific primers and normalized against actin. **L & M.** Measurement of nuclear localization of NRF2 after lapatinib and tucatinib treatment. Following the co-pretreatment strategy, MDA-MB-453 cells were exposed to lapatinib for 1h **(L)** and tucatinib for 36h **(M)**. Immunofluorescence assays were performed to detect the nuclear translocation of NRF2 (red), counterstained with DAPI (blue). Images were captured at 100X using a confocal microscope. **N & O.** Evaluation of nuclear localization of NRF2 in a time-dependent manner post-lapatinib and tucatinib treatment. MDA-MB-453 cells were treated with 3.5 μ M lapatinib for 1, 3, 6, 10, and 24h **(N)** and 5 μ M tucatinib for 12, 24, 36, 40 and 48h **(O)** followed by an immunofluorescence assay to detect the nuclear localization of NRF2 (red), counterstained with DAPI (blue). **P & Q.** Evaluation of correlation between mitochondrial ROS production, IGF-1, IGF-1R and nuclear NRF2 expression by HER2-positive breast cancer cells after lapatinib and tucatinib treatment. As mentioned in Figure 4J-K, along with mitochondrial ROS, IGF-1 and IGF-1R expression, nuclear NRF2 expression was also measured at the same time points following lapatinib **(P)** and tucatinib **(Q)** treatment. **R.** Correlation analysis between NRF2, IGF-1, IGF-1R and mitochondrial ROS post-lapatinib and tucatinib treatment using Python code. **S & T.** Measurement of pFOXO1/3a protein expression post-lapatinib and tucatinib exposure. As described, whole-cell lysates were harvested, lysed, and subjected to immunoblot analysis with pFOXO1/3a-specific antibody after lapatinib **(S)** and tucatinib **(T)** exposure. GAPDH was used as a loading control.

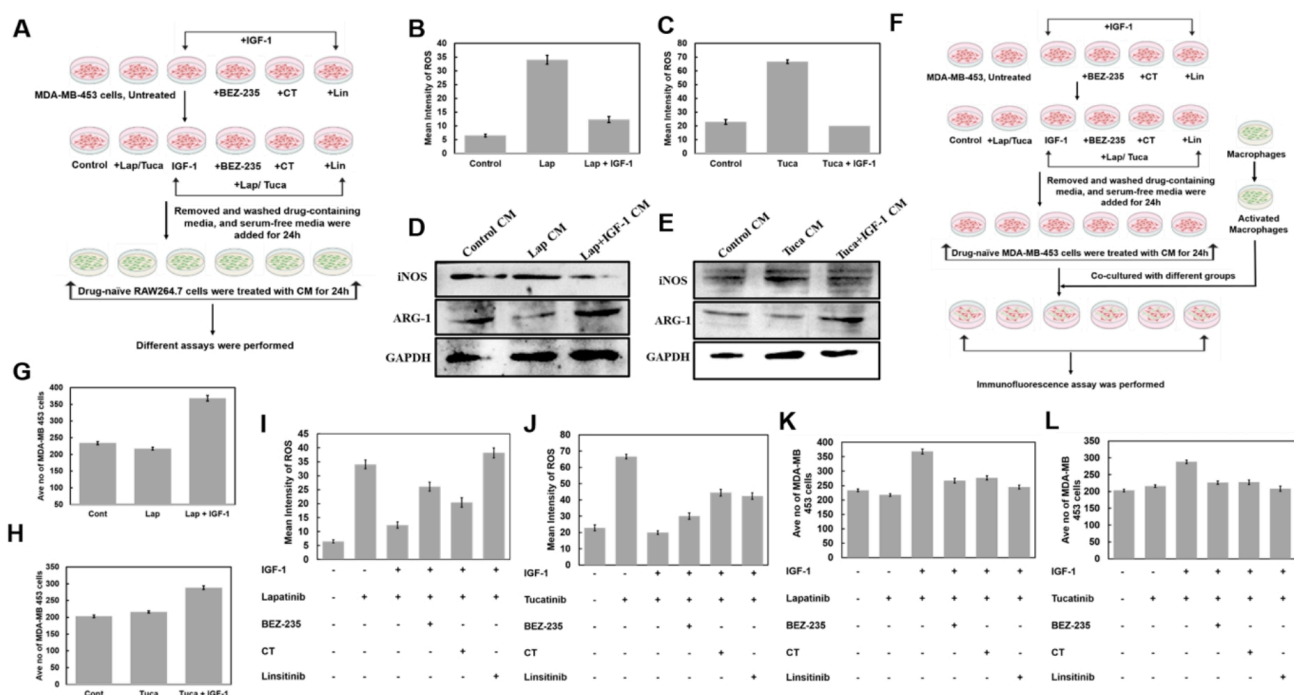


Figure 5

Lapatinib and tucatinib promote macrophage polarization to the M1 phenotype, but this shift is reversed to M2 by IGF-1/PI3K/NRF2 signaling. **A.** Experimental design for assessing the impact of lapatinib and tucatinib-treated (with or without IGF-1) cancer cells on RAW264.7 macrophage polarization. MDA-MB-453 cells were divided into seven groups: two untreated and five pretreated with 50ng/ml IGF-1 alone or in combination with 100nM BEZ-235, 250nM camptothecin (CT), or 40μM linsitinib for 6h. Cells were then exposed to 3.5μM lapatinib or 5μM tucatinib for 24h, incubated in serum-free medium for 24h, and then collected conditioned medium (CM). Drug naïve RAW264.7 macrophages were then exposed to the CM for 24h before performing various assays. **B & C.** Assessment of mitochondrial ROS production in macrophages. Following 24h treatment with CM from lapatinib (**B**) and tucatinib (**C**)-treated MDA-MB-453 cells, mitochondrial ROS formation was assessed in RAW264.7 cells using MitoSox dye. **D & E.** Measurement of iNOS and ARG-1 protein levels in macrophages. Following 24h treatment with CM from lapatinib (**D**) and tucatinib (**E**)-treated MDA-MB-453 cells, RAW264.7 cells were lysed and subjected to immunoblot analyses for iNOS (M1-specific marker) and ARG-1 (M2-specific marker). GAPDH was used as a loading control. **F.** Experimental design for assessing the impact of lapatinib and tucatinib-treated (with or without IGF-1) cancer cells on macrophage-mediated immune clearance. MDA-MB-453 cells were divided into seven groups: two untreated and five pretreated with IGF-1 alone or in combination with BEZ-235, camptothecin (CT), or linsitinib for 6h. Following pretreatment, cells were exposed to 3.5μM lapatinib or 5μM tucatinib for 24h, then incubated in serum-free medium for 24h to collect conditioned medium (CM). Drug-naïve MDA-MB-453 cells labeled with CellTracker Orange

were treated with the CM collected for 24h and co-cultured with RAW264.7 macrophages activated with LPS and IFN- γ , labeled with CellTracker Green, at a 1:1 ratio for 48h before performing immunofluorescence assays. **G & H.** Measurement of MDA-MB-453 cell number post-lapatinib CM (**G**) and tucatinib CM (**H**) treatment after co-culturing with activated macrophages for immune clearance. **Using** this strategy, we counted the average number of MDA-MB-453 cells. **I & J.** Assessment of mitochondrial ROS production by RAW264.7 macrophages after pharmacological blockage of PI3K, NRF2 and IGF-1R. After treating the macrophages with CM from lapatinib (**I**) and tucatinib (**J**) plus/minus the above-mentioned inhibitor-treated MDA-MB-453 cells (as outlined in A), mitochondrial ROS production was assessed using the MitoSox dye. **K & L.** Measurement of RAW264.7 macrophage-mediated immune clearance of MDA-MB-453 cells exposed to CM from lapatinib (**K**) and tucatinib (**L**) plus/minus PI3K, NRF2, and IGF-1R inhibitor-treated cells (as mentioned in F), the average number of MDA-MB-453 cells was counted.

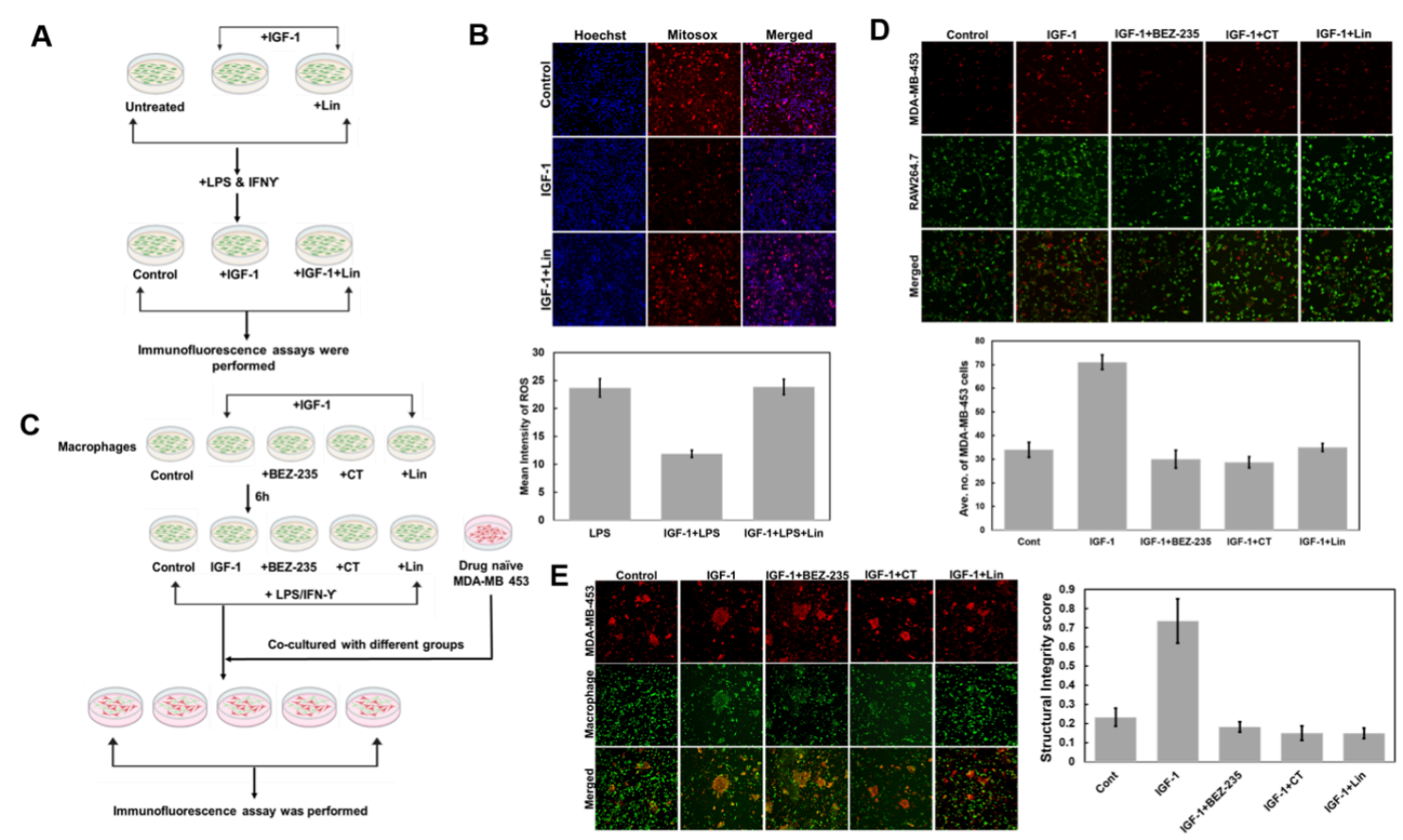


Figure 6

IGF-1 directly alters macrophage polarization and protects against the clearance of cancer cells in a PI3K/NRF2-dependent manner. **A.** Experimental design for the assessment of ROS generation post-macrophage activation. RAW264.7 macrophages were divided into three groups: one untreated and two pretreated with 50ng/ml IGF-1 alone or with 40μM linsitinib, followed by activation with LPS and IFN- γ . These cells were then subjected to an immunofluorescence assay. **B.** Assessment of mitochondrial ROS. Following the strategy mentioned in A, mitochondrial ROS in RAW264.7 macrophages with IGF-1

plus/minus linsitinib treatment was measured using the MitoSox dye. **C.** Experimental design for assessing RAW264.7 macrophage-mediated clearance of cancer cells. RAW264.7 macrophages were divided into five groups; one untreated and four treated with IGF-1 alone or in combination with IGF-1R/PI3K/ NRF2 inhibitors for 6h. Cells were then activated with LPS and IFN-g for 24h and tracked with CellTracker Green. Drug naïve MDA-MB-453 cells were labeled with cell tracker orange and co-cultured with activated RAW264.7 macrophages in a 1:1 ratio for 48h, after which immunofluorescence assay was performed. **D.** Assessment of clearance of cancer cells co-cultured with IGF-1-treated macrophages with or without treatment with inhibitors of the IGF-1R/PI3K/NRF2 axis. As mentioned in the strategy in C, the average number of CellTracker Orange-labeled MDA-MB-453 cells was counted after co-culturing with activated macrophages (treated with IGF-1 and IGF-1R/PI3K/NRF2 inhibitors) to check for immune clearance. **E.** Assessment of IGF-1 plus/minus IGF-1R/PI3K/NRF2 inhibitor-treated MDA-MB-453 organoid clearance from activated RAW264.7 macrophages.

The p-values among different comparisons are included in Supplemental Table 9.

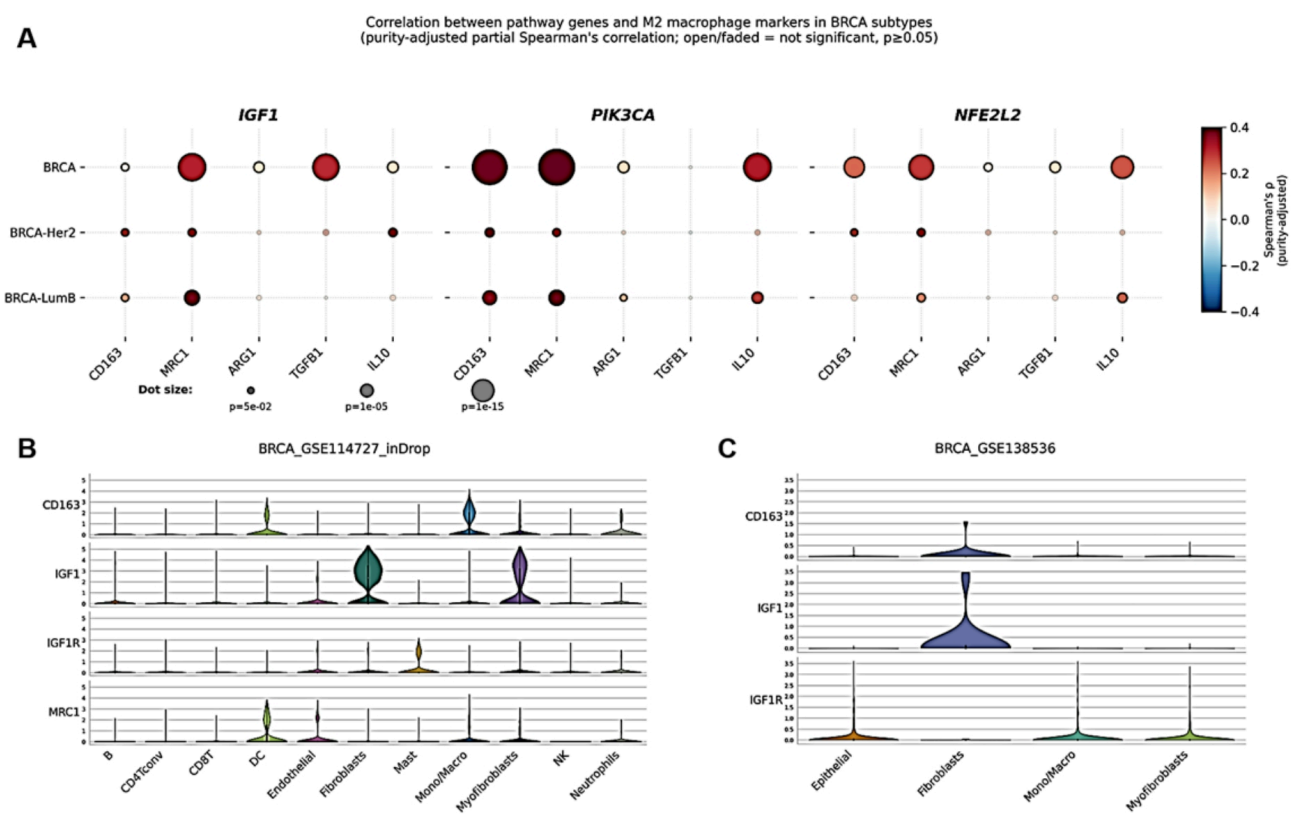


Figure 7

Clinical correlation between the IGF-1/PI3K/NRF2 pathway and M2 macrophage infiltration with single-cell validation of pathway component expression. **A.** Correlation between IGF-1, PIK3CA (encoding the PI3K catalytic subunit), and NFE2L2 (NRF2) expression and canonical M2 macrophage markers (CD163,

MRC1/CD206, ARG1, TGFB1, IL10) in the TCGA breast invasive carcinoma (BRCA) cohort, analyzed using TIMER2.0. Correlations were computed as purity-adjusted partial Spearman's correlations for the pooled BRCA cohort and the BRCA-Her2 and BRCA-LumB molecular subtypes. Dot color represents Spearman's ρ (red, positive; blue, negative); dot size represents statistical significance (larger dots indicate smaller p-values, as shown in the size legend). Faded/open dots indicate non-significant correlations ($p \geq 0.05$). **B-C.** Single-cell RNA-seq expression of CD163, IGF-1, IGF-1R, and MRC1 (**B**) or CD163, IGF-1, and IGF-1R (**C**) across major cell types in two independent human breast cancer datasets, BRCA_GSE114727_inDrop (**B**) and BRCA_GSE138536 (**C**), visualized using TISCH2. Violin plots show the expression distribution of each gene within each annotated cell type.

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