

# TNF $\alpha$ -Induced Mucin 4 Expression Elicits Trastuzumab Resistance in HER2-Positive Breast Cancer

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## Abstract

**Purpose:** Although trastuzumab administration improved the outcome of HER2-positive breast cancer patients, resistance events hamper its clinical benefits. We demonstrated that TNF $\alpha$  stimulation *in vitro* induces trastuzumab resistance in HER2-positive breast cancer cell lines. Here, we explored the mechanism of TNF $\alpha$ -induced trastuzumab resistance and the therapeutic strategies to overcome it.

**Experimental Design:** Trastuzumab-sensitive breast cancer cells, genetically engineered to stably overexpress TNF $\alpha$ , and *de novo* trastuzumab-resistant tumors, were used to evaluate trastuzumab response and TNF $\alpha$ -blocking antibodies effectiveness respectively. Immunohistochemistry and antibody-dependent cell cytotoxicity (ADCC), together with siRNA strategy, were used to explore TNF $\alpha$  influence on the expression and function of its downstream target, mucin 4 (MUC4). The clinical relevance of MUC4 expression was studied in a cohort of 78 HER2-positive breast cancer patients treated with adjuvant trastuzumab.

**Results:** TNF $\alpha$  overexpression turned trastuzumab-sensitive cells and tumors into resistant ones. Histopathologic findings revealed mucin foci in TNF $\alpha$ -producing tumors. TNF $\alpha$  induced upregulation of MUC4 that reduced trastuzumab binding to its epitope and impaired ADCC. Silencing MUC4 enhanced trastuzumab binding, increased ADCC, and overcame trastuzumab and trastuzumab-emtansine antiproliferative effects in TNF $\alpha$ -overexpressing cells. Accordingly, administration of TNF $\alpha$ -blocking antibodies downregulated MUC4 and sensitized *de novo* trastuzumab-resistant breast cancer cells and tumors to trastuzumab. In HER2-positive breast cancer samples, MUC4 expression was found to be an independent predictor of poor disease-free survival ( $P = 0.008$ ).

**Conclusions:** We identified TNF $\alpha$ -induced MUC4 expression as a novel trastuzumab resistance mechanism. We propose MUC4 expression as a predictive biomarker of trastuzumab efficacy and a guide to combination therapy of TNF $\alpha$ -blocking antibodies with trastuzumab. *Clin Cancer Res*; 1–13. ©2016 AACR.

## Introduction

HER2/neu overexpression/amplification occurs in approximately 20% of invasive breast cancers and is strongly associated

with poor prognosis (1). Trastuzumab, the first clinically approved mAb against HER2, is the standard-of-care treatment for patients with early and metastatic HER2-positive breast cancer administered in combination with chemotherapy (2). Its mechanism of action relies mainly on inhibiting HER2-mediated signal transduction and triggering antibody-dependent cell-mediated cytotoxicity (ADCC; refs. 3, 4). The administration of trastuzumab profoundly improved the outcome of HER2-positive breast cancer patients. However, up to 42% of the patients treated with neoadjuvant trastuzumab and 27% of those with adjuvant trastuzumab experience disease progression (5, 6). The lack of sustained response can be due to *de novo* or acquired resistance (7). Multiple mechanisms underlying trastuzumab resistance in breast cancer have been described, including persistent activation of the PI3K–Akt pathway (8), cross-talk of heterologous receptor signaling pathways (9, 10) and cleavage of HER2 extracellular domain (11), among others. Trastuzumab-emtansine (T-DM1), a novel HER2-targeting antibody–drug conjugate, was recently approved as a second-line treatment for advanced HER2-positive breast cancer patients who progressed to trastuzumab-based therapy (12). T-DM1 combines the HER2-targeting properties and mechanism of action of trastuzumab with the selective delivery of DM1 (a potent derivative of the anti-microtubule agent maytansine) to HER2-positive breast cancer cells. The

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

M.F. Mercogliano and M. De Martino contributed equally to this article.

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## Translational Relevance

Trastuzumab is given to breast cancer patients with no biomarker indication other than HER2 overexpression or amplification. Resistance to therapy is a frequent life-threatening event in patients. To date, there are no diagnostic procedures to prevent/overcome resistance or to guide tailored-made treatments. In our work, using TNF $\alpha$ -overexpressing and *de novo* trastuzumab-resistant cell lines, we disclosed TNF $\alpha$  as a novel source of trastuzumab and trastuzumab-embtansine resistance, acting through the induction of MUC4 expression. Results showed that trastuzumab-resistant cells expressed higher TNF $\alpha$  levels than trastuzumab-sensitive ones. The combined blockade of HER2 and TNF $\alpha$  overcame *de novo* trastuzumab resistance. Our findings highlight MUC4 expression as a prognostic biomarker of trastuzumab response and suggest that MUC4- and HER2-positive breast cancer patients could benefit from a combination of TNF $\alpha$ -blocking antibodies with trastuzumab.

response rate in patients with advanced breast cancer who received T-DM1 is between 26% and 58% (13). However, the mechanisms underlying T-DM1 resistance have been poorly characterized (14).

Mucin 4 (MUC4) is a glycoprotein that belongs to the membrane-bound family of mucins and has two non-covalently associated subunits encoded by a single gene (15). The extracellular subunit, MUC4 $\alpha$ , is heavily glycosylated and provides antiadhesive properties to the cell. The transmembrane subunit MUC4 $\beta$ , contains two EGF-like domains in the extracellular portion that can interact with HER2 (16). MUC4 is normally expressed in the apical region of mammary cells. In breast cancer cells, it is aberrantly expressed in the cytosol and shows an increased expression pattern in metastatic lesions, compared to primary-matched tumors (17). In addition, MUC4 has been reported to mask the trastuzumab-binding epitope of HER2, thereby decreasing its binding *in vitro* in JIMT-1, a *de novo* trastuzumab-resistant breast cancer cell line (18). Nevertheless, regulation of MUC4 expression by endogenous mediators and its relevance on trastuzumab resistance *in vivo* remains to be addressed.

The presence of inflammatory mediators in the tumor micro-environment, either generated by the tumor cells or by tumor-infiltrating cells, is widely recognized in cancer biology (19, 20). In particular, low-grade chronic inflammation plays a key role in the pathogenesis and progression of breast cancer (21). Indeed, inflammation has been associated with poor prognosis in breast cancer patients and with an increased risk of recurrence (22, 23). Moreover, NF- $\kappa$ B signaling, the main transcription factor induced by inflammatory mediators, such as cytokines, is activated in cells resistant to anti-estrogen therapies (24). However, the participation of proinflammatory cytokines in trastuzumab resistance has been poorly explored. Our earlier study characterized TNF $\alpha$  as a non-canonical activator of HER2. We demonstrated that stimulation of HER2-positive breast cancer cells *in vitro* with TNF $\alpha$  induces HER2 transactivation, which in turn activates NF- $\kappa$ B and induces proliferation of trastuzumab-sensitive cell lines, even in the presence of this mAb (25).

Here, we revealed that TNF $\alpha$  is a key player in trastuzumab resistance in HER2-positive breast cancer *in vivo*. Using *de novo* trastuzumab-resistant breast cancer xenografts and genetically engineered cells to stably overexpress TNF $\alpha$ , we elucidated that the mechanism of resistance relies on TNF $\alpha$ -induced MUC4 expression. Remarkably, we also found that MUC4 is a source of T-DM1 resistance. Consistent with these results, we observed that MUC4 expression is associated with reduced disease-free survival (DFS) and is an independent predictor of poor outcome for HER2-positive breast cancer patients treated with adjuvant trastuzumab. These findings provide the rationale for a combinatorial therapy of trastuzumab and TNF $\alpha$ -blocking antibodies, which could be administered to patients with MUC4- and TNF $\alpha$ -positive tumors, to overcome or prevent trastuzumab resistance in the clinical setting.

## Materials and Methods

### Cell lines

SK-BR-3, MDA-MB-453, and NCI-N87 cells were obtained from the ATCC and JIMT-1 cells from the German Resource Centre for Biological Material. KPL-4 and BT-474 cells were kindly provided by Dr. J. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan) and Dr. D. Yu (The University of Texas, MD Anderson Cancer Center, Houston, TX), respectively. BT-474 T1 and T2 cells were developed by transducing BT-474 cells with Open Biosystems Precision LentiORF Collection lentiviral particles containing the open reading frame of the human TNF $\alpha$  and BT-474 C cells with Thermo Scientific Precision LentiORF TurboRFP Control Particles (Thermo Scientific). Cells were cultured in presence of 5.5  $\mu$ g/mL blasticidine (Santa Cruz Biotechnology). Experiments and cell maintenance were performed in DMEM: F12, RPMI1640, or McCoy medium supplemented with 10% FBS. All of the above mentioned cell lines were authenticated by STR profiling at GRFCF, Johns Hopkins University (Baltimore, MD). Cells were routinely tested for Mycoplasma contamination.

### Immunoblotting, flow cytometry, antibodies, and reagents

Immunoblotting was performed as described previously (26), with details in Supplementary Data. Flow cytometry assay, antibodies, and reagents are described in Supplementary Data.

### ELISA

Human TNF $\alpha$  concentration was measured in tumor extracts, serum, or supernatants by ELISA (PeproTech), following the manufacturer's specifications.

### Proliferation assays

Cell proliferation was evaluated either by [<sup>3</sup>H]-thymidine (NEN, DuPont; specific activity: 20 Ci/mmol) incorporation assay at 48 hours of culture with an 18-hour pulse, as we previously described (26) or by cell count in Neubauer chamber with Trypan blue (25).

### Transient transfections

siRNAs targeting MUC4 were synthesized by Dharmacon and the ones targeting NF- $\kappa$ B p65 were from Cell Signaling Technology. Protein expression was analyzed 48 hours after transfection. Details of transfection are described in Supplementary Data.

### ADCC assay

Immediately before the assay,  $10^6$  BT-474 C and T2 cells (target cells) were incubated with 50  $\mu$ Ci  $^{51}$ Chromium ( $^{51}\text{CrO}_4\text{Na}_2$ , NEN, DuPont) for 1 hour. Then, they were co-cultured with human peripheral blood mononuclear cells, at different target:effector ratios, in the presence of 10  $\mu$ g/mL trastuzumab or rituximab for 4 hours at 37°C. Cell death was determined by  $^{51}\text{Cr}$  release in a gamma counter. Specific cell cytotoxicity was calculated as ( $^{51}\text{Cr}$  released by target cells –  $^{51}\text{Cr}$  released by normal cells)/(maximum  $^{51}\text{Cr}$  released –  $^{51}\text{Cr}$  released by normal cells).

### In vivo experiments

Female NIH(S)-nude mice (La Plata National University, Argentina; female, 8–10-weeks-old) bearing an 0.72-mg estradiol pellet, were subcutaneously injected with  $10^7$  BT-474 C, T1, or T2 cells resuspended in 1:1 v/v DMEM:F12:Matrigel (Becton Dickinson). Once tumors were established (volume of 50–70 mm $^3$ ), animals were randomly allocated to receive intraperitoneal injection of 5 mg/kg trastuzumab or human IgG twice a week. Nude mice were subcutaneously injected with  $3 \times 10^6$  JIMT-1 or KPL-4 cells. When tumors were established, animals received either 5 mg/kg etanercept once a week, 5 mg/kg trastuzumab twice a week, etanercept plus trastuzumab, or 5 mg/kg human IgG twice a week. Tumor growth was measured routinely with a Vernier caliper and volume was calculated as described previously (26). All animal studies were conducted in accordance with the highest standards of animal care as outlined by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the IBYME Animal Research Committee.

### Patients

Breast cancer paraffin-embedded tissue sections of 78 consecutive patients with HER2-positive primary invasive ductal breast cancer were retrieved from the Pathology Department of Hospital Fernández, Instituto Oncológico Henry Moore (Buenos Aires, Argentina) and Hospital Temuco (Temuco, Chile) from 2005 to 2014. The median follow-up time was of 30 months (range, 0.5–9 years). Also a cohort of 72 patients with HER2-negative ER-positive primary invasive ductal breast cancer was included in the analysis. This study was conducted according to the provisions of the Declaration of Helsinki and informed written consents were obtained from all patients before inclusion. The study protocols were approved by the Ethical Committees of the participating institutions. Patients were included if they had received adjuvant trastuzumab treatment, had complete data on baseline clinical features and treatment outcomes, and were preoperatively chemotherapy and radiotherapy naïve. The tumor specimens were anonymized for this study. Clinicopathologic data of the cohorts are shown in Supplementary Table S1.

### Histopathologic analysis and IHC

For IHC, antigen retrieval was performed in 10 mmol/L sodium citrate buffer pH 6 for 50 minutes at 92°C in paraffin-embedded tissue sections. Slides were incubated with antibodies against TNF $\alpha$  (Abcam; #9739, 1:100) and MUC4 (1G8, Santa Cruz Biotechnology, 1:50) overnight at 4°C. Sections were incubated with HRP system (Vector Laboratories) and developed with DAB (Cell Marque). A score of 0 to 3+ was used as previously reported (17). Quantification details of TNF $\alpha$ , MUC4, HER2, estrogen, and progesterone receptor determination are described in Supplemen-

tary Data. Guidelines for Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) were followed in this work.

### Statistical analysis

Statistical differences were determined by two-tailed *t* test, one-way ANOVA coupled with a Tukey *post hoc* test or two-way ANOVA coupled with a Bonferroni *post hoc* test using the GraphPad Prism 6 Program (GraphPad Software).

Statistical significance was analyzed using Statistical Package SPSS version 15 for Windows (SPSS Inc.). The  $\chi^2$  test was used to analyze MUC4 association with clinicopathologic characteristics of the tumors. DFS was calculated from the date of initial diagnosis to the date of recurrence or death, whichever came first. Cumulative DFS probabilities were calculated according to the Kaplan–Meier method and statistical significance was analyzed by log-rank test. Covariates that were statistically significant in a univariate model were included in a multivariate analysis using the Cox proportional hazards regression model. The HR and its 95% confidence interval (CI) were calculated for each variable. *P* values less than 0.05 were considered statistically significant and all reported *P* values were two sided.

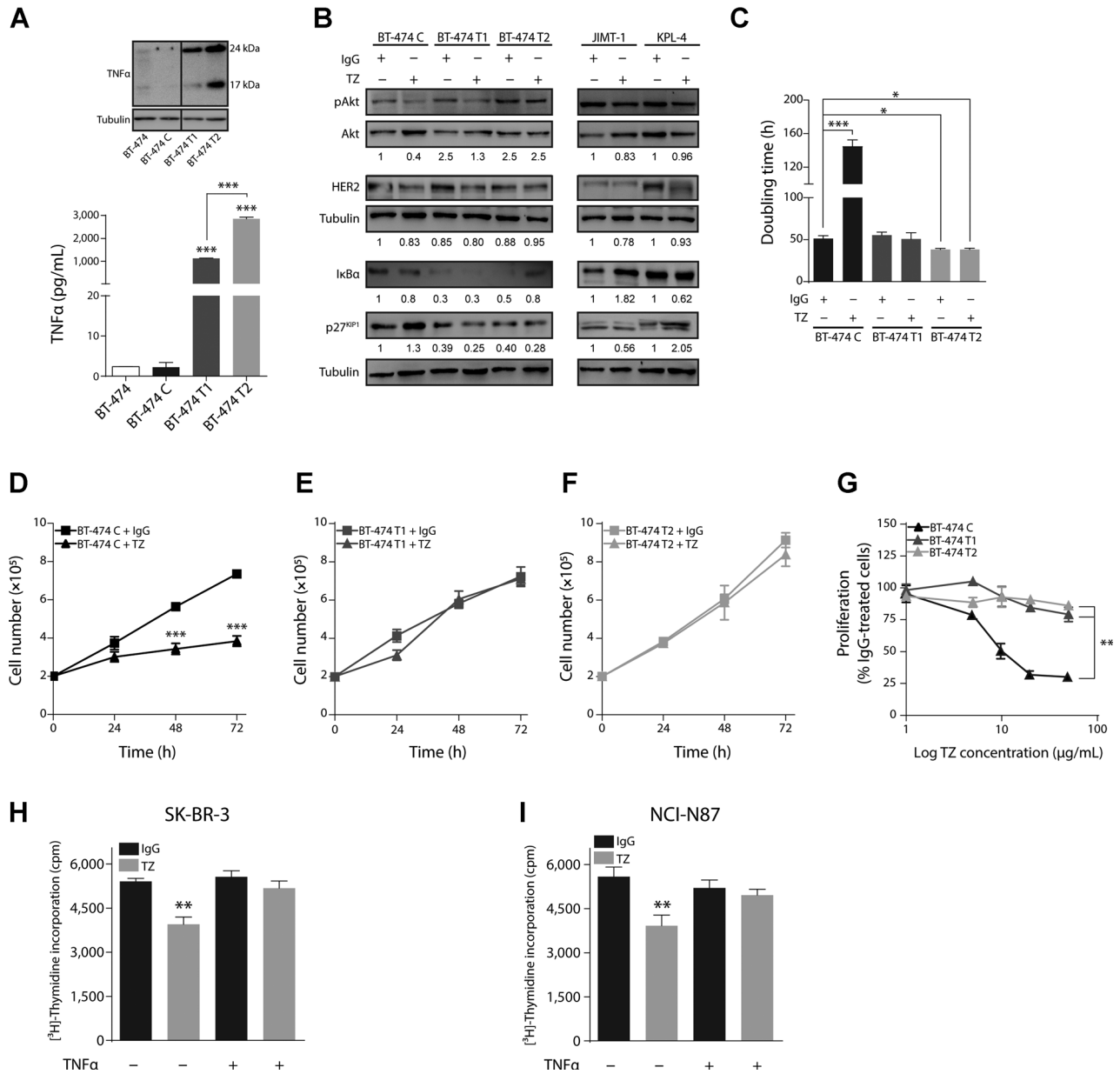
## Results

### TNF $\alpha$ confers trastuzumab resistance in HER2-positive breast and gastric cancer

We previously reported that TNF $\alpha$  enhances *in vitro* proliferation of BT-474 cells, a trastuzumab-sensitive HER2-positive human breast cancer cell line and showed that they did not respond to the antiproliferative effect of this antibody (25). To explore the *in vitro* participation of constitutive TNF $\alpha$  expression in trastuzumab resistance, we transduced BT-474 cells with lentiviral particles encoding human TNF $\alpha$  (BT-474 T1 and T2 cells) or containing an empty vector (BT-474 C cells). Figure 1A shows TNF $\alpha$  expression by immunoblot and ELISA in these cell lines and in the parental cells.

Activation of the PI3K–Akt pathway induces resistance to trastuzumab treatment (6). Then, we explored phospho-Akt (S473) levels in the BT-474 cell variants after 24 hours of trastuzumab treatment. Trastuzumab treatment strongly inhibited phosphorylation of Akt in BT-474 C cells, but it had no effect on BT-474 T2 cells. Although, BT-474 T1 cells treated with trastuzumab showed a decrease in phospho-Akt levels with respect to IgG-treated cells but were still higher than that of BT-474 C treated with IgG. JIMT-1 and KPL-4, two *de novo* trastuzumab-resistant cell lines (27, 28), exhibit higher levels of phospho-Akt than BT-474 C treated with trastuzumab (Fig. 1B). HER2 protein expression remained unchanged in all three cell lines (Fig. 1B). TNF $\alpha$ -expressing BT-474 cells exhibited lower levels of I $\kappa$ B $\alpha$  expression than BT-474 C cells, indicating NF- $\kappa$ B pathway activation even in the presence of trastuzumab (Fig. 1B). Another hallmark of effective trastuzumab treatment is the upregulation of the cell-cycle inhibitor p27 $^{\text{KIP1}}$ . Here, we found that trastuzumab treatment increased p27 $^{\text{KIP1}}$  levels in BT-474 C cells, but had no effect in either BT-474 T1 or T2 cells (Fig. 1B).

BT-474 T2 cells displayed a higher proliferation rate and a shorter doubling time than BT-474 C and T1 cells even in the presence of 10  $\mu$ g/mL trastuzumab (Fig. 1C–F). Trastuzumab inhibited proliferation of BT-474 C cells (Fig. 1C and D). BT-474 T1 cells exhibited similar proliferation rate and doubling time compared with BT-474 C cells, but were not affected by



**Figure 1.**

TNFα confers resistance to trastuzumab in HER2-positive breast and gastric cancer. **A**, BT-474 cells were stably transduced with lentiviral particles containing a human TNFα expression vector (BT-474 T1 and T2) or particles with an empty vector (BT-474 C). TNFα expression is shown by immunoblot of cell lysates and by ELISA in cell supernatants. **B**, Levels of activated Akt, HER2, IκBα and p27<sup>KIP1</sup> in BT-474 C, T1 and T2 cells treated with 10 μg/mL trastuzumab or IgG for 24 hours were analyzed by immunoblots of cell lysates. Similarly, the *de novo* trastuzumab-resistant KPL-4 and JIMT-1 cell lines treated with IgG and trastuzumab were evaluated. Numbers below panels indicate the densitometric analysis of pAkt/Akt, HER2, IκBα, and p27<sup>KIP1</sup>/tubulin ratios with respect to IgG-treated BT-474 C cells, in the case of *de novo* trastuzumab-resistant cell lines the ratios were done in reference to IgG-treated JIMT-1 or KPL-4. **C**, Doubling time was calculated based on data shown in **D-F**. **D-F**, Proliferation of BT-474 C (**D**), T1 (**E**) and T2 cells (**F**) treated with 10 μg/mL trastuzumab or IgG was determined at 24, 48, and 72 hours by cell count. **G**, Trastuzumab sensitivity of BT-474 C, T1, and T2 cells was determined at 72 hours after treatment with 1, 5, 10, 20, and 50 μg/mL of the antibody. Data are represented as percentages of cells treated with trastuzumab with respect to cells treated with IgG. **H** and **I**, SK-BR-3 and NCI-N87 cells were preincubated for 24 hours with 20 ng/mL TNFα and then 10 μg/mL trastuzumab or IgG was added to the culture. Proliferation was determined at 48 hours by [<sup>3</sup>H]-thymidine incorporation with an 18-hour pulse. **A**, **C** data represent mean ± SD (*n* = 3), and in **H** and **I** *n* = 6 and were analyzed using one-way ANOVA coupled with a Tukey *post hoc* test. **D-G**, Data represent mean ± SD (*n* = 3). Data were analyzed using two-way ANOVA coupled with a Bonferroni *post hoc* test; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001. TZ, trastuzumab.

trastuzumab treatment (Fig. 1C and E). Sensitivity to trastuzumab was assessed through a concentration–response curve at 72 hours posttreatment. Notably, trastuzumab achieved the highest inhi-

bition of BT-474 C proliferation at a concentration of 20 μg/mL, but BT-474 T1 and T2 cells remained unaffected even at a concentration of 50 μg/mL trastuzumab (Fig. 1G). Together, these

data confirm that BT-474 cells became resistant to trastuzumab upon overexpression of TNF $\alpha$ .

To further validate the *in vitro* effects of TNF $\alpha$  on trastuzumab resistance, we stimulated trastuzumab-sensitive, HER2-overexpressing cell lines SK-BR-3 (breast) and NCI-N87 (gastric) cell lines with this proinflammatory cytokine. We observed that TNF $\alpha$  is able to confer resistance to the antiproliferative effects of trastuzumab in both cell lines (Fig. 1H and I, respectively). These findings underscore that TNF $\alpha$  confers resistance to trastuzumab in HER2-positive human breast and gastric cancer cells.

To test whether BT-474 T1 and T2 retain trastuzumab resistance *in vivo*, we subcutaneously injected these cells in nude mice and compared their growth with that of BT-474 C cells (Supplementary Table S2). When tumors reached a volume of 50–70 mm<sup>3</sup>, mice were randomly allocated to receive trastuzumab or human IgG treatment. BT-474 T2 tumors displayed a statistically non-significant trend towards higher tumor growth rate than its BT-474 C counterpart ( $P = 0.07$ ; Supplementary Table S2). Trastuzumab administration induced a striking regression of BT-474 C tumor, consistent with the report regarding the parental BT-474 cell line (ref. 29; Fig. 2A). In contrast, trastuzumab treatment of animals bearing BT-474 T2 or T1 tumors did not change their growth rate (Fig. 2A; Supplementary Table S2). Tumor weights were in line with these results (Fig. 2B). TNF $\alpha$  concentration was higher in tumor extracts of BT-474 T2 tumors and in the serum of animals bearing this tumor than in BT-474 C tumor and serum counterpart (Fig. 2C and D). Histopathologic analysis of the BT-474 T2 tumors treated with IgG or trastuzumab, showed high-grade tumors with aggressive features such as muscle and dermis infiltration (Supplementary Fig. S1A and S1B). An important peritumor leukocyte infiltration was also present (Supplementary Fig. S1C). Similar findings to those obtained in BT-474 T2 were observed in BT-474 T1 tumors (Supplementary Fig. S1D). Extensive areas of fibrosis (70%–90%) were observed in BT-474 C tumors treated with trastuzumab (Fig. 2E). Detection of HER2 by IHC and by immunoblots of tumor extracts, showed comparable expression levels in BT-474 C, T1, and T2 tumors (Fig. 2E and F and Supplementary Fig. S1D), consistent with the *in vitro* findings shown in Fig. 1B. In addition, phospho-Akt levels were higher in BT-474 T2 tumors compared with BT-474 C tumors, and remained unchanged upon trastuzumab treatment (Fig. 2F). These proof-of-principle experiments showed that TNF $\alpha$  is an important cytokine in mediating *in vivo* trastuzumab resistance in human HER2-positive breast cancer.

#### TNF $\alpha$ blockade overcomes *de novo* trastuzumab resistance in HER2-positive breast tumors

*De novo* trastuzumab resistance arises in up to 60% of the patients, when administered with chemotherapy (6, 30). We determined that three *de novo* trastuzumab-resistant cell lines, JIMT-1, KPL4, and MDA-MB-453 secrete higher levels of TNF $\alpha$  compared with trastuzumab-sensitive SK-BR-3, BT-474 and NCI-N87 cells (Fig. 3A). This result led us to explore TNF $\alpha$  participation in *de novo* trastuzumab resistance. Proliferation of JIMT-1 and KPL-4 cells was evaluated in the presence or absence of 10  $\mu$ g/mL trastuzumab with the addition of different concentrations of etanercept, a TNFR2-IgG fusion protein that blocks TNF $\alpha$  functions. Etanercept alone did not show any effect on JIMT-1 cell proliferation at 10  $\mu$ g/mL, but significantly inhibited proliferation at 20 or 30  $\mu$ g/mL (Fig. 3B). Trastuzumab did not affect cell growth as a single agent. However, simultaneous addition of both

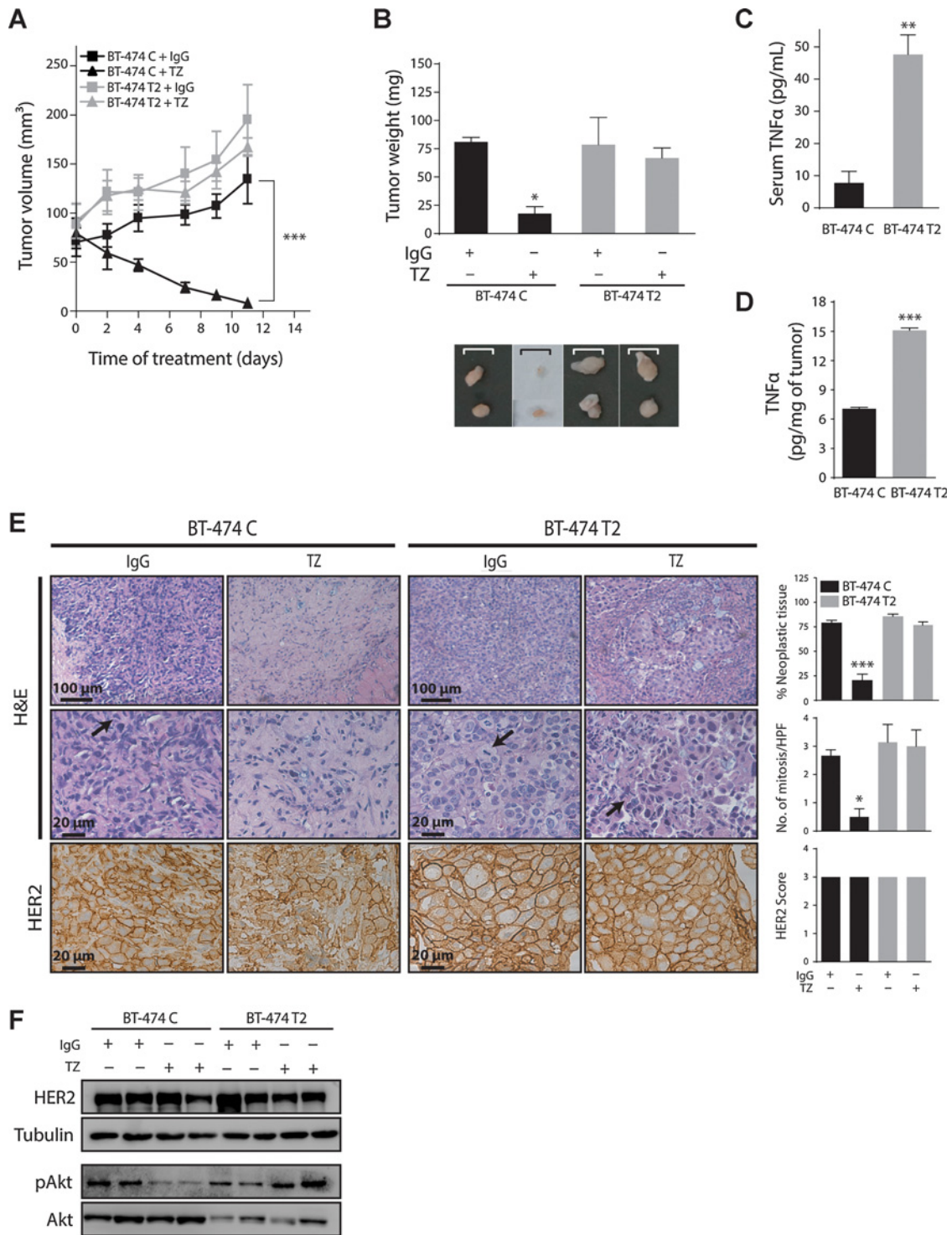
antibodies decreased JIMT-1 cell proliferation in a synergistic manner (Fig. 3B). In KPL-4 cells, etanercept alone was able to inhibit proliferation at 30  $\mu$ g/mL. Trastuzumab treatment in combination with 10, 20, or 30  $\mu$ g/mL etanercept reduced KPL-4 cell proliferation significantly (Fig. 3B). These results encouraged us to evaluate the efficacy of trastuzumab plus etanercept treatment in a preclinical setting. To this end, JIMT-1 or KPL-4 cells were injected into nude mice, and when tumors reached 50 to 70 mm<sup>3</sup>, mice were administered with 5 mg/kg trastuzumab twice a week, 5 mg/kg etanercept weekly or both agents simultaneously. A group was injected with human IgG as control. Tumor growth rate was not affected by trastuzumab or etanercept single-agent treatments, compared with tumors from the IgG-treated group. However, the combination of trastuzumab and etanercept caused significant inhibition of tumor growth in JIMT-1 xenografts and regression of KPL-4 tumors, confirmed by tumor weights at the end of the experiment (Fig. 3C and D, respectively; Supplementary Table S3).

Histopathologic analysis showed that JIMT-1 tumors from mice treated with trastuzumab and etanercept presented hyalinization (Fig. 3E), an indicator of regressive disease. KPL-4 tumors that received trastuzumab and etanercept showed areas with hyalinization and extensive necrosis (Fig. 3E). These data provide evidence that TNF $\alpha$  blockade can be a suitable therapy to overcome *de novo* trastuzumab resistance.

#### TNF $\alpha$ induces MUC4 expression in HER2-positive breast and gastric cancer cells

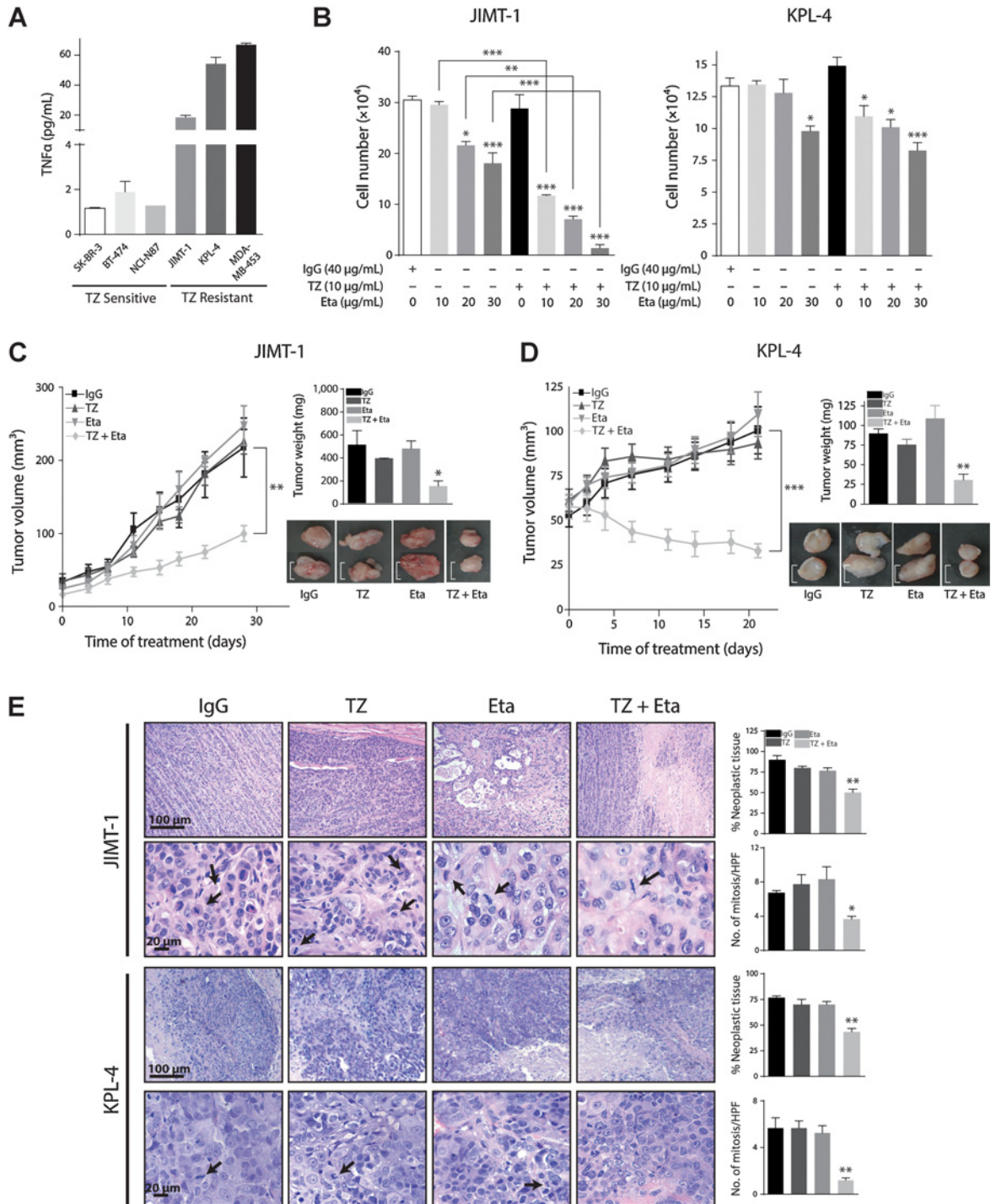
One of the proposed *in vitro* mechanisms of trastuzumab resistance in JIMT-1 cells is linked to MUC4 expression, which hinders the epitope of trastuzumab on the HER2 molecule (18). Interestingly, hematoxylin and eosin (H&E) specimen examination of BT-474 T2 tumors, either treated with IgG or trastuzumab, revealed the presence of mucin vacuoles in the cytoplasm of the tumor cells (Fig. 4A). We thus evaluated whether TNF $\alpha$  could be a positive regulator of MUC4 expression, which would explain trastuzumab resistance in these tumors. We observed a stronger staining of MUC4 by IHC in BT-474 T2 tumors compared with BT-474 C tumors, which was confirmed by immunoblots of tumor extracts (Fig. 4B, MUC4 scores are described in Supplementary Fig. S2). In addition, JIMT-1 and KPL-4 tumors from mice treated with IgG or trastuzumab showed strong cytoplasmic staining for MUC4 (Fig. 4C). On the other hand, tumors from mice receiving etanercept alone or in combination with trastuzumab showed weak to undetectable staining for MUC4 (Fig. 4C). *In vitro* constitutive TNF $\alpha$  expression resulted in 1.4- and 3.6-fold induction of MUC4 levels in BT-474 T1 and T2 cells, respectively, compared with those of the BT-474 C cells (Fig. 4D). In addition, TNF $\alpha$  treatment of SK-BR-3 and NCI-N87 cells increased MUC4 expression in both cell lines (Fig. 4E) that can be related to trastuzumab resistance induced by TNF $\alpha$  shown in Fig. 1H and I.

We previously demonstrated that TNF $\alpha$  induces NF- $\kappa$ B pathway activation in trastuzumab-sensitive HER2-positive cells even in the presence of this antibody (25). To determine the participation of NF- $\kappa$ B in TNF $\alpha$ -driven MUC4 expression, we silenced NF- $\kappa$ B p65 in BT-474 C, T1, and T2 cells (Fig. 4F) and in SK-BR-3 and NCI-N87 treated or not with TNF $\alpha$  (Fig. 4G). Abrogation of NF- $\kappa$ B p65 expression abolished TNF $\alpha$ -induced MUC4 upregulation in BT-474 T1, T2, SK-BR-3, and NCI-N87 cells. Together, our

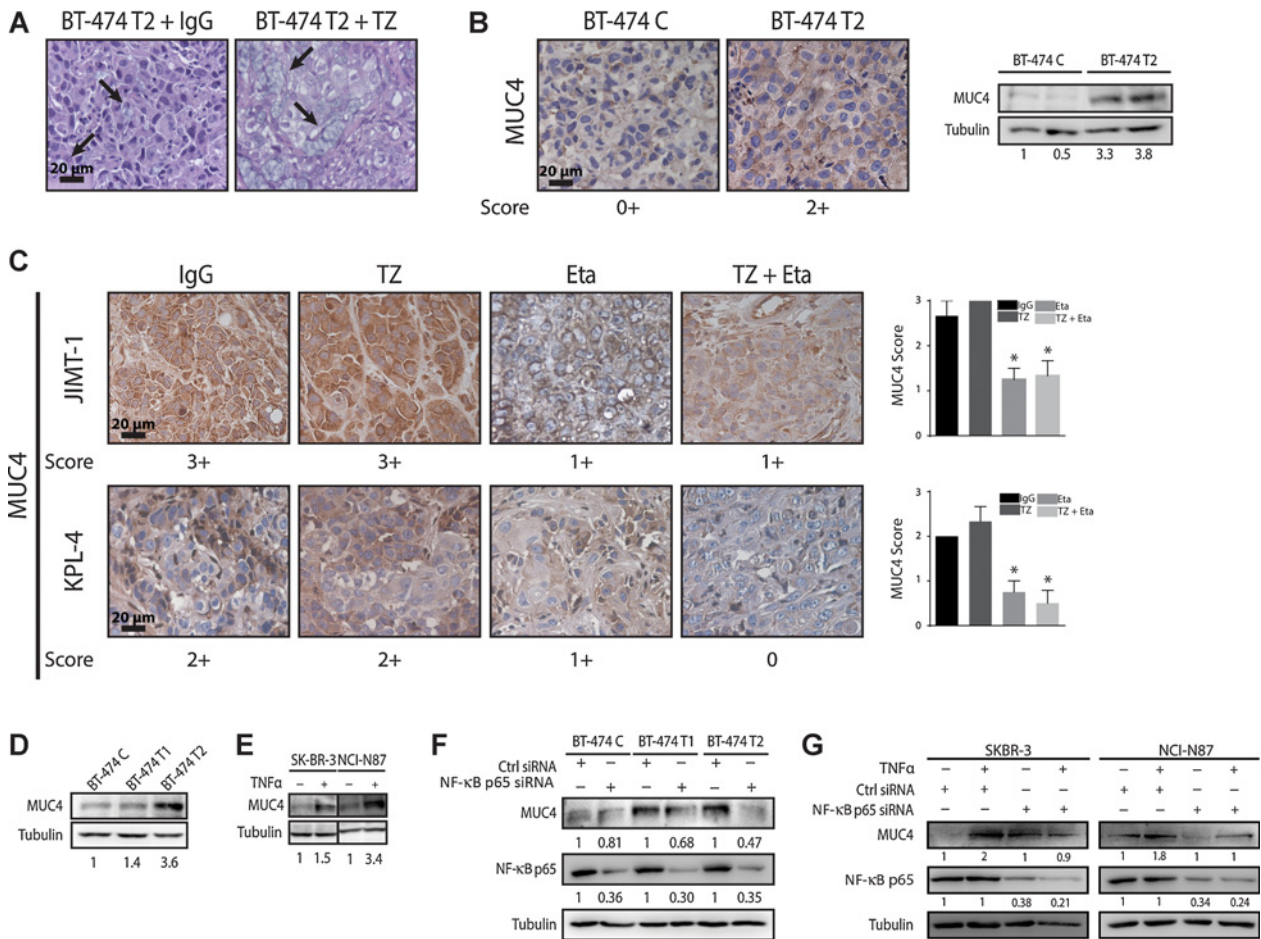


**Figure 2.**

TNF $\alpha$ -producing tumors are resistant to trastuzumab administration. Nude mice were injected with BT-474 C or T2 cells. When tumors were established, animals were treated with 5 mg/kg of trastuzumab or with human IgG twice a week. **A**, Tumor growth is shown as tumor volume  $\pm$  SD ( $n = 6$  to  $8$ ). **B**, At the end of the experiment, tumors were weighed and photographed; scale bars, 1 cm. **C** and **D**, Determination of human TNF $\alpha$  serum levels in tumor-bearing mice and in tumor extracts by ELISA, respectively. Data represent mean  $\pm$  SD ( $n = 5$ ). **E**, Histopathologic analysis of tumors. Quantification of the following parameters is shown in the right: First, the percentage of neoplastic tissue of the entire section by H&E staining; second, proliferation by mitotic figures count per HPF; third, HER2 score by IHC. Each data point represents mean  $\pm$  SD ( $n = 6$ ). Arrows indicate mitotic figures. **F**, Immunoblots of HER2 and phospho-Akt from representative tumor lysates are shown. **A**, **C**, and **D**, Data were analyzed using two-tailed nonpaired  $t$  test and in **B** and **E** by one-way ANOVA coupled with a Tukey *post hoc* test \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**Figure 3.** Simultaneous treatment with an anti-TNF $\alpha$  antibody and trastuzumab effectively inhibits growth of *de novo* trastuzumab-resistant tumors. **A**, TNF $\alpha$  determination by ELISA of supernatants of SK-BR-3, BT-474, NCI-N87, JIMT-1, KPL-4, and MDA-MB-453 cells. Data represent mean  $\pm$  SD ( $n = 3$ ). **B**, Proliferation of JIMT-1 and KPL-4 cells treated with 10  $\mu$ g/mL trastuzumab, different concentrations of etanercept or both antibodies was determined by cell count at 72 hours. Data represent mean  $\pm$  SD ( $n = 3$ ). **C** and **D**, Nude mice were injected with JIMT-1 or KPL-4 cells, respectively. Once tumors were established, animals were administrated with 5 mg/kg trastuzumab twice a week, 5 mg/kg etanercept once a week or both antibodies. The control group was injected with 5 mg/kg human IgG. Tumor growth is shown as tumor volume  $\pm$  SD ( $n = 5-6$ ) in the left graph. At the end of the experiment, tumors were weighed (right top) and photographed (right bottom); scale bars, 1 cm. **E**, Histopathologic analysis of tumors. Right, the quantification of the percentage of neoplastic tissue of the entire section by H&E staining (first and third lines) and proliferation by mitotic figures count per HPF (second and fourth lines). Arrows indicate mitotic figures. Each data point represents mean  $\pm$  SD ( $n = 5$ ). **B-E**, Data were analyzed by one-way ANOVA coupled with a Tukey *post hoc* test. **C** and **D**, Growth curves were analyzed by two-tailed nonpaired *t* test; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Eta, etanercept.



**Figure 4.**

TNF $\alpha$  induces MUC4 expression *in vivo* and *in vitro* in breast cancer cells. **A**, H&E staining of BT-474 T2 tumors treated with IgG or trastuzumab, as described in Fig. 2. Arrows show mucinous foci. **B**, IHC studies of MUC4 expression in BT-474 C and T2 tumors treated with IgG as depicted in Fig. 2 (left). MUC4 score is shown below the figures and was obtained as shown in Supplementary Fig. S2. Immunoblot of MUC4 in representative tumor lysates (right). **C**, IHC of MUC4 in JIMT-1 and KPL-4 tumors treated with IgG, trastuzumab, etanercept, or trastuzumab plus etanercept, as described in Fig. 3. **D** and **E**, MUC4 levels of BT-474 C, T1 and T2 cells in complete media and of SK-BR-3 and NCI-N87 cells treated with 20 ng/mL TNF $\alpha$  for 24 hours were assessed in cell lysates by immunoblots. **F** and **G**, BT-474 C, T1, T2 cells (**F**) and SK-BR-3 and NCI-N87 cells (**G**) were transfected with siRNA against NF- $\kappa$ B p65 or control siRNA (Ctrl siRNA) for 48 hours. SK-BR-3 and NCI-N87 cells were then treated with 20 ng/mL TNF $\alpha$  for 24 hours. MUC4 and NF- $\kappa$ B p65 expression levels were assessed in cell lysates by immunoblot. Numbers below panels indicate the densitometric analysis of MUC4/tubulin and NF- $\kappa$ B p65/tubulin with respect to control cells. **C**, Data were analyzed by one-way ANOVA coupled with a Tukey *post hoc* test; \*,  $P < 0.05$ .

data demonstrate that TNF $\alpha$ , acting through the NF- $\kappa$ B pathway, induces an increase in MUC4 expression in HER2-positive breast cancer cells.

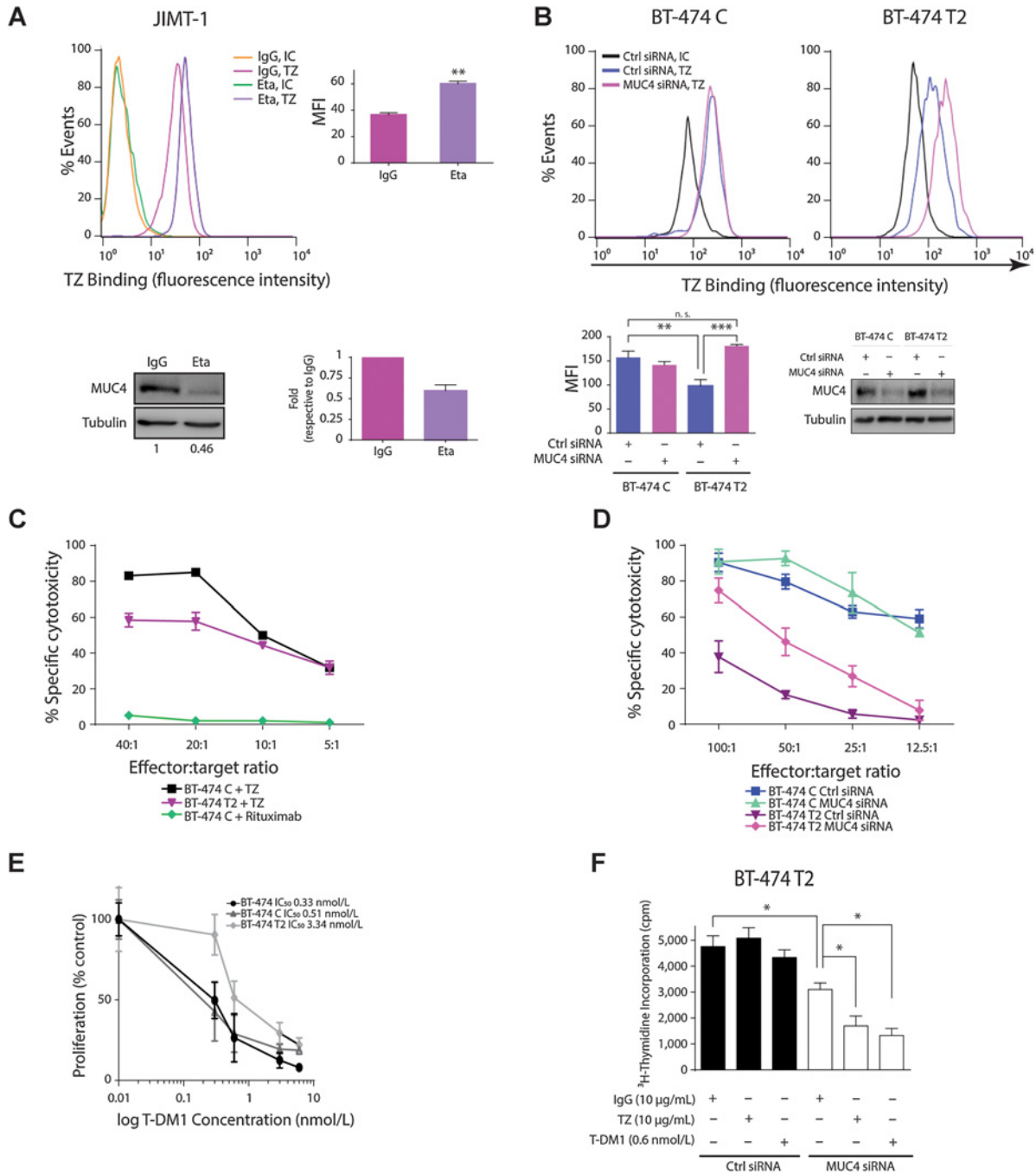
**TNF $\alpha$ -induced MUC4 expression inhibits trastuzumab-induced cell cytotoxicity and also impairs T-DM1 antiproliferative effect in HER2-positive breast cancer cells**

Our findings strongly suggest that induction of MUC4 expression by TNF $\alpha$  could be an important mechanism of trastuzumab resistance. We thus evaluated trastuzumab binding in JIMT-1 cells after etanercept treatment. To achieve this, we incubated JIMT-1 cells with etanercept or IgG for 24 hours, and then performed an indirect immunofluorescence assay using trastuzumab as the primary antibody. Flow cytometry analysis revealed higher trastuzumab binding in etanercept-treated cells than in the IgG-treated counterparts (Fig. 5A). We also observed a reduc-

tion in MUC4 expression by immunoblot in JIMT-1 cells treated with etanercept (Fig. 5A). Moreover, BT-474 T2 cells showed lower trastuzumab-binding capacity than BT-474 C cells. However, MUC4 silencing in BT-474 T2 cells restored trastuzumab binding, achieving levels comparable with the ones in BT-474 C cells (Fig. 5B).

Because ADCC is one of the main mechanisms of trastuzumab action *in vivo* (4), we examined the sensitivity of TNF $\alpha$ -producing breast cancer cells to ADCC using BT-474 C and T2 cells as target cells. A strong ADCC activity (~80%) on BT-474 C cells was observed with trastuzumab meanwhile BT-474 T2 cells were less susceptible to trastuzumab-induced cell cytotoxicity (~60%; Fig. 5C). To determine whether MUC4 induction was responsible for BT-474 T2 resistance to ADCC, we silenced MUC4. MUC4 knock-down partially restored ADCC susceptibility of BT-474 T2 cells, compared with BT-474 C cells (Fig. 5D). Considering the fact that





**Figure 5.**

TNF $\alpha$ -induced MUC4 expression hinders trastuzumab binding and antibody-dependent cell cytotoxicity, and inhibits antiproliferative effect of T-DM1 in HER2-positive breast cancer cells. **A**, Binding of trastuzumab was determined in JIMT-1 cells treated with 5  $\mu$ g/mL etanercept or human IgG for 24 hours, by indirect immunofluorescence and flow cytometry and quantification of trastuzumab binding by mean fluorescence intensity (top). MUC4 expression in JIMT-1 cell lysates by immunoblot and the quantification of five independent experiments (bottom). **B**, Binding of trastuzumab in BT-474 C (top left) and T2 cells (top right) transfected with control siRNA or siRNA targeting MUC4 for 48 hours. Quantification of trastuzumab binding by mean fluorescence intensity (bottom left) is shown (mean  $\pm$  SD;  $n = 3$ ). Effectiveness of MUC4 knockdown is shown (bottom right). **C**, Trastuzumab-mediated cell cytotoxicity. BT-474 C and T2 cells (target cells, loaded with <sup>51</sup>Cr) were cocultured with human peripheral blood mononuclear cells (effector cells) at different target-to-effector cells ratios in presence of 10  $\mu$ g/mL trastuzumab or rituximab (control) for 4 hours. Cytotoxicity was determined by <sup>51</sup>Cr release assay. **D**, Trastuzumab-mediated cell cytotoxicity in BT-474 C and T2 cells transfected with control siRNA or MUC4 siRNA. **E**, Cell proliferation of BT-474 C and T2 cells treated with T-DM1 for 48 hours at different concentrations was determined by [<sup>3</sup>H]-thymidine incorporation with an 18-hour pulse. **F**, BT-474 T2 cells were transfected with control siRNA or MUC4 siRNA and then treated with trastuzumab, T-DM1, or IgG. Cell proliferation was determined as in **E**. **A-D**, Data represent mean  $\pm$  SD ( $n = 3$ ). **E** and **F**, Each data point represents mean  $\pm$  SD ( $n = 6$ ). **A**, Data were analyzed using the two-tailed *t* test. **B** and **F**, data were analyzed using one-way ANOVA coupled with a Tukey *post hoc* test. **E**, IC<sub>50</sub> values were determined from four-parameter curve fitting. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . MFI, mean fluorescence intensity; IC<sub>50</sub>, half maximal inhibitory concentration.

T-DM1 has an enhanced therapeutic effect compared with trastuzumab, we examined the effect of TNF $\alpha$  on this treatment using cell proliferation as readout. Both BT-474 and BT-474 C cells were sensitive to low concentrations of T-DM1 with IC<sub>50</sub> 0.33 nmol/L (0.055  $\mu$ g/mL) and 0.51 nmol/L (0.085  $\mu$ g/mL), respectively (Fig. 5E). Meanwhile, in BT-474 T2 cells, T-DM1 was approximately 10 times less potent than in parental and control cells [IC<sub>50</sub> 3.34 nmol/L (0.55  $\mu$ g/mL; Fig. 5E)]. These findings led us to study the role of MUC4 on resistance to T-DM1 in BT-474 T2 cells. We observed that MUC4 knockdown dramatically inhibited proliferation of BT-474 T2 cells in the presence of trastuzumab or T-DM1 (Fig. 5F). Taken together, these data highlight TNF $\alpha$ -induced MUC4 expression as a hindrance for trastuzumab binding to HER2 and the consequent reduction in the effectiveness of ADCC in HER2-positive breast cancer cells. We also demonstrated that MUC4 is also responsible for T-DM1 resistance.

#### MUC4 expression is a predictive biomarker of response to trastuzumab in HER2-positive breast cancer patients

To address the clinical relevance of our findings, we examined TNF $\alpha$  and MUC4 expression by IHC in a cohort of 78 HER2-positive primary invasive breast cancers obtained before adjuvant trastuzumab treatment. Examination of the HER2-positive cohort showed TNF $\alpha$  and MUC4 positivity in 89% and 60% of the tumor samples, respectively (Fig. 6A and Supplementary Fig. S2; Supplementary Table S4). We found a positive correlation between MUC4 and TNF $\alpha$  expression ( $P < 0.04$ ; Supplementary Table S4). Although TNF $\alpha$  expression by itself did not correlate with the outcome of trastuzumab treatment (data not shown), Kaplan–Meier analysis revealed that tumors coexpressing TNF $\alpha$  and MUC4 were associated with impaired DFS with respect to TNF $\alpha$ -positive/MUC4-negative tumors ( $P = 0.016$ ; Fig. 6B). When we explored the clinical relevance of MUC4 expression, we observed that MUC4-positive tumors exhibited a statistically strong association with shorter DFS in patients receiving adjuvant trastuzumab-based therapy ( $P = 0.0086$ ). The mean DFS was 54 months [95% confidence interval (CI), 49–60 months] for MUC4-negative and 47 months (95% CI, 42–52 months) for MUC4-positive tumors (Fig. 6C). The median DFS was 55 months (95% CI, 43–66 months) for MUC4-positive patients, whereas the median DFS for the MUC4-negative patients was not reached (Fig. 6C). No association between MUC4 expression and standard clinicopathologic characteristics was observed (Supplementary Table S5). Then, we tested MUC4 expression in a cohort of 72 breast cancer patients that were not treated with trastuzumab, that belonged to the luminal subtype. Kaplan–Meier analysis showed that MUC4 expression was not associated with patients' outcome of this cohort (Supplementary Fig. S3). This evidence strength our findings that MUC4 can be used as a specific biomarker of trastuzumab response in HER2-positive breast cancer.

We then investigated whether the clinical outcome of the HER2-positive cohort was associated with various clinicopathologic parameters, including MUC4 status. In univariate analysis, MUC4-positive tumors were associated with significantly shorter DFS than MUC4-negative ones (HR, 4.40; 95% CI, 1.28–15.09;  $P = 0.018$ ). Higher T stage (HR, 5.25; 95% CI, 2.09–13.18;  $P = 0.0004$ ), N stage (2.56; 95% CI, 1.02–6.43;  $P = 0.045$ ), and clinical stage (HR, 5.47; 95% CI, 2.14–13.95;  $P = 0.0004$ ) were also associated with short DFS (Fig. 6D). Multivariate analysis (Fig. 6E) adjusted for T and node status, identified MUC4 as an independent predictor of poor DFS, with an increased HR of 5.43

(95% CI, 1.56–18.80;  $P = 0.008$ ). These data suggest that MUC4 could serve as a biomarker to predict HER2-positive breast cancer survival of patients treated with trastuzumab in the adjuvant setting.

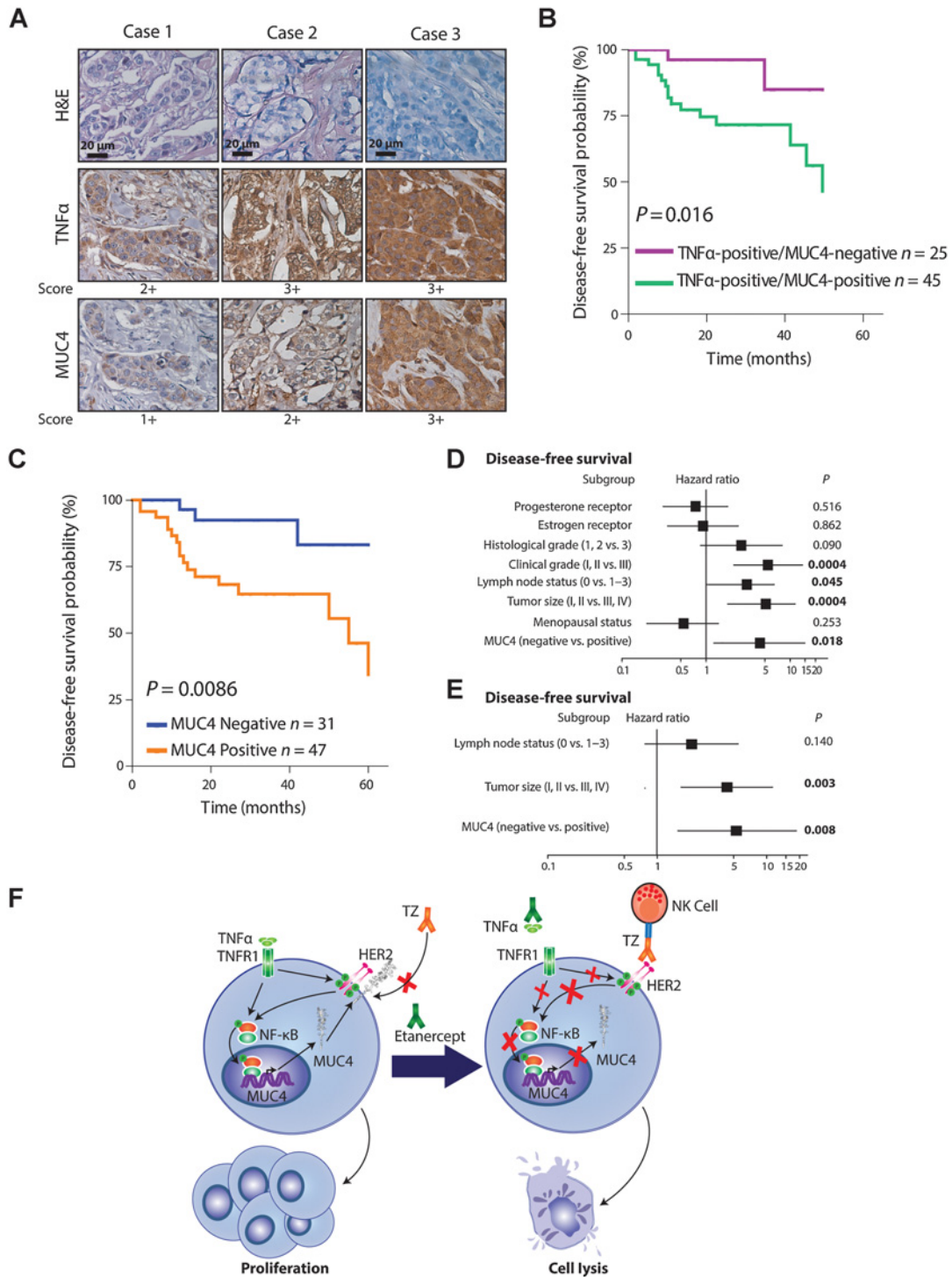
## Discussion

In the current report, we identified TNF $\alpha$  as a novel contributor to trastuzumab resistance in HER2-positive breast cancer. TNF $\alpha$  induced cell proliferation that was not inhibited by trastuzumab treatment *in vitro* and *in vivo*. We disclosed that TNF $\alpha$  induced elevated expression levels of MUC4, that mask the trastuzumab-binding epitope on HER2, thereby impeding the antiproliferative effect and ADCC action of trastuzumab. Administration of TNF $\alpha$ -blocking antibodies *in vivo* overcame trastuzumab resistance in *de novo* resistant tumors and induced MUC4 downregulation. The proposed model is depicted in Fig. 6F. Moreover, we revealed that MUC4 expression also contributes to T-DM1 resistance.

Constitutive activation of NF- $\kappa$ B was observed in HER2-positive breast cancer specimens (31) and it was reported that induced resistance to HER2-targeted therapies (32, 33). To our knowledge, this study is the first to demonstrate that TNF $\alpha$ , the major cytokine able to activate NF- $\kappa$ B, can modify trastuzumab sensitivity of HER2-positive breast and gastric cell lines. TNF $\alpha$  overexpression turns trastuzumab-sensitive cells and tumors into resistant ones. We demonstrated that these cells exhibited high levels of Akt activation, comparable with those in *de novo* trastuzumab-resistant cell lines. In addition, *de novo* trastuzumab-resistant cells showed higher levels of secreted TNF $\alpha$  than trastuzumab-sensitive cells, and TNF $\alpha$  blockade sensitized them to the therapeutic action of trastuzumab. Our data suggest that TNF $\alpha$  is a possible druggable target to prevent or overcome trastuzumab resistance in the clinic.

A significant finding in our work was the demonstration that TNF $\alpha$  is able to upregulate MUC4 expression *in vitro* and *in vivo*. Although the precise molecular mechanisms underlying the regulation of MUC4 are not completely understood, some cytokines and growth factors are known to regulate its expression. In gastric and breast cancer cells, it was reported that IL6, fibronectin, and EGF are able to induce MUC4 expression through STAT3 activation (34). Here, we described that NF- $\kappa$ B is essential for TNF $\alpha$ -induced MUC4 upregulation in HER2-positive breast cancer cells. Some studies have provided evidence regarding MUC4 involvement in trastuzumab resistance *in vitro*. The expression of rat Muc4 in MCF-7 cells or endogenous MUC4 in JIMT-1 cells, induced a reduction in trastuzumab binding, caused by a steric hindrance of the interaction between trastuzumab and its epitope on HER2 (18, 35). Although upregulation of MUC4 was also found in a xenograft breast cancer model resistant to anti-HER2 therapies, the functional consequences of MUC4 overexpression were not explored in said study (36). Here, we observed that MUC4 silencing successfully unmasked the epitope of trastuzumab in HER2 in TNF $\alpha$ -overexpressing cells, thereby restoring trastuzumab binding and ADCC activities. Also, we determined that TNF $\alpha$  blockade with etanercept decreased MUC4 expression *in vitro* and *in vivo*, while it enhanced trastuzumab binding in *de novo* trastuzumab-resistant cells.

It is known that T-DM1 binds to HER2 with a similar affinity as trastuzumab. Recognition of the epitope on the HER2 molecule is required for T-DM1 to exert its antitumor activity. Consistent with this notion, our findings show that TNF $\alpha$ -induced MUC4



**Figure 6.**

MUC4 expression correlates with poor response to adjuvant trastuzumab treatment in HER2-positive breast cancer patients. **A**, A total of 78 HER2-positive breast cancer specimens were analyzed by IHC at diagnosis. Representative H&E and IHC studies of TNF $\alpha$  and MUC4 are shown. Tumors were considered MUC4- or TNF $\alpha$ -positive when they exhibited a score of 2+ or 3+. **B** and **C**, Kaplan-Meier analysis of the probability of DFS of patients with HER2- and TNF $\alpha$ -positive tumors (**B**) or with HER2-positive tumors (**C**), who received adjuvant trastuzumab treatment, based on the expression of MUC4. **D** and **E**, Forest plots showing the HRs (squares) and 95% CI (horizontal lines) of univariate and multivariate subgroup analysis, respectively. **F**, Working model of TNF $\alpha$ -induced trastuzumab resistance. TNF $\alpha$ , from tumor cell or microenvironment, induces transactivation of HER2 (25), and activation of NF- $\kappa$ B, promoting MUC4 expression. MUC4, in turn, shields trastuzumab-binding epitope on HER2, decreasing or blocking its antitumor effect. TNF $\alpha$  blockade with etanercept abrogates these effects, allowing trastuzumab action and consequent ADCC mediated by NK cells. **B** and **C**, The  $P$  value was calculated using the log-rank test. **D** and **E**, Data were analyzed using Cox univariate and multivariate proportional hazards regression models, respectively.

expression is also able to block the antiproliferative effects of trastuzumab and T-DM1. To explore a possible tool to predict T-DM1 efficacy in metastatic patients, a study compared pretreatment imaging of HER2 with zirconium-89–radiolabeled trastuzumab ( $^{89}\text{Zr}$ [T] uptake using PET-CT (37). The authors found that HER2-imaging-positive patients have a larger time-to-treatment failure (TTP) than those who were negative. A recent report showed that the use of a mucolytic agent could enhance  $^{89}\text{Zr}$ [T] accumulation and improve the HER2 imaging in JIMT-1 tumor xenografts (38). Taken these lines of evidence as a whole, we can hypothesize that the low-to-null uptake of  $^{89}\text{Zr}$ [T] observed in poor TTP patients could be due, at least in part, to MUC4-positive breast cancers.

Notably, our study in a patient cohort of 78 HER2-positive primary tumors showed that TNF $\alpha$  is present in most of the samples evaluated, confirming previous reports (39), and highlighted its relevance in the breast cancer microenvironment. Although TNF $\alpha$  can promote immune surveillance that aims to eliminate tumors, it can also drive chronic inflammation, denoted by the presence of M2 macrophages, promote growth and spreading of breast cancer cells, reflecting that its role on the immune response is contextual and pleiotropic (22). Several concordant studies indicate that disease outcome in patients with HER2-positive breast cancer treated with adjuvant or neoadjuvant trastuzumab improves when tumors exhibit an abundant immune infiltrate (40, 41), the impact of M2 macrophage infiltration and response to trastuzumab in the clinical setting remains unknown. In this regard, a recent report demonstrated that intratumor injection of IL-21 sensitized a breast cancer HER2-positive xenograft to trastuzumab by inducing polarization of tumor-associated macrophages from M2 to M1 phenotype (42).

Currently, trastuzumab is given to patients with no biomarker indication other than HER2 overexpression or amplification. Therefore, the development of predictive biomarkers is essential for the administration of a suitable and effective therapy. Here, we demonstrated for the first time that MUC4 expression in HER2-positive breast cancer is associated with reduced DFS and is an independent predictor of poor outcome in patients treated with trastuzumab in the adjuvant setting. It is worth mentioning that the sensitivity of prediction of relapse using MUC4 expression was high (91% of patients who relapsed had MUC4-positive tumors). We propose that the determination of MUC4 expression by IHC, as a companion biomarker, will be able to reveal a subgroup of patients that would not benefit from trastuzumab or T-DM1 treatment, which should undergo more frequent clinical controls and/or additional therapeutic intervention. In this respect, TNF $\alpha$ -blocking antibodies have been used in the clinic for over 10 years in patients suffering from rheumatoid arthritis. The experience indicates that among patients with rheumatoid arthritis and a history of breast cancer, those who started anti-TNF $\alpha$  treatment did not have more breast cancer recurrence than patients with

rheumatoid arthritis treated otherwise (43). In addition, etanercept was used in a phase II clinical trial in metastatic breast cancer in which it was reported safe and well tolerated (44), making it *a priori* feasible to use in combination with trastuzumab.

In conclusion, this is the first report to demonstrate that TNF $\alpha$  plays a central role in trastuzumab resistance by acting through the TNF $\alpha$ –NF- $\kappa$ B–MUC4 axis. We propose that MUC4 expression could be used as a biomarker of trastuzumab responsiveness, leading to personalized cancer treatment protocols involving TNF $\alpha$  inhibitors in combination with trastuzumab. Forthcoming clinical trials should reveal the efficacy of the proposed therapy.

## Disclosure of Potential Conflicts of Interest

I. Frahm is a consultant/advisory board member for Roche. No potential conflicts of interest were disclosed by the other authors.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M.F. Mercogliano, M. De Martino, L. Venturutti, M.A. Rivas, G. Inurrigarro, E.G. Deza, F.G. Gercovich, P. Guzmán, P.V. Elizalde, R. Schillaci

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