Intratumoral Cytokines and Tumor Cell Biology Determine Spontaneous Breast Cancer–Specific Immune Responses and Their Correlation to Prognosis

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Abstract

Spontaneous immune responses in cancer patients have been described. Yet their clinical relevance and the conditions for their generation remain unclear. We characterized conditions that determine immune responses in primary breast cancer patients. We used tetramer analysis, ex vivo IFN-γ ELISPOT, cytotoxicity assays, and ELISA in 207 untreated patients and 12 Her-2/neu–specific CD8 T-cell lines to evaluate tumor-specific T cells (TC) in the bone marrow or MUC1-specific antibodies in the blood. Multiplex analysis was performed to quantify 27 intratumoral cytokines, chemokines, and growth factors. Results were compared with multiple pathologic and clinical parameters of the patients and tumors. Forty percent of the patients showed tumor-specific TC responses. These correlated with tumors of high differentiation, estrogen receptor expression, and low proliferative activity, and with a reduced cancer mortality risk. High tumor cell differentiation correlated with increased intratumoral, but not plasma, concentrations of IFN-α and reduced transforming growth factor (TGF)β1. In an in vitro priming experiment these two cytokines increased or inhibited, respectively, the capacity of dendritic cells to induce tumor-reactive TC. Tumor-specific B-cell responses, mainly of IgM isotype, were detectable in 50% of the patients and correlated with advanced tumor stage, increased TGFβ1, reduced IFN-α, and absence of TC responses. We show here that different types of immune responses are linked to distinct cytokine microenvironments and correlate with prognosis-relevant differences in tumor pathobiology. These findings shed light on the relation between immune response and cancer prognosis. [Cancer Res 2009;69(21):8420–8]

Introduction

Spontaneous T-cell (TC) responses are increasingly recognized as beneficial prognostic factors in cancer. For example, increased TC infiltration is a major prognostic factor in colorectal cancer and associated with improved prognosis in breast cancer (1–3). Tumor antigen (TA)–specific memory TC from breast cancer patients mediated complete rejections of xenotransplanted autologous tumors (4, 5). These findings suggest a protective role of tumor-specific TC during tumor progression or relapse. Spontaneous TC responses against TA have been reported in a broad variety of tumors (4–9). TA-specific CD8 TC in the peripheral blood (PB) of patients are often anergic, whereas functionally competent TC are enriched in the bone marrow (BM; refs. 4, 5, 8, 10), which is specialized in recruitment and accumulation of memory TC and a site for the induction of primary TC responses against TA (11, 12). Approximately 60% of breast cancer patients develop spontaneous tumor-reactive memory TC in their BM (3, 4), and immune infiltrates have been detected frequently in breast cancer lesions and are correlated with improved prognosis (2, 3). To date, the conditions required for the spontaneous generation of TA-specific TC remain largely unclear.

We here used the repertoire of TC in the BM of primary untreated, nonmetastasized breast cancer patients to characterize features of patients or their tumors that are associated with the spontaneous generation of TA-specific immune responses and to evaluate their potential prognostic implications.

Materials and Methods

Patients and healthy donors. BM and PB samples were taken after informed consent from primary breast cancer patients during surgery or from 30 healthy females, and mononuclear cells were collected as described (4). None of the patients had received neoadjuvant treatment. Tumor pieces and sera were obtained during surgery and snap frozen.

HLA-A2 typing. HLA-A2[+] expression was analyzed using anti–HLA-A2 monoclonal antibody BB7.2 (BD Pharmingen) as described (8).

Cell lines and antigens. MCF7 mammary carcinoma cells and U937 leukemia cells were used (4). The TAP-deficient HLA-A*0201[+] T2 cell line (4) was used for loading with HLA-A*0201–restricted peptides: MUC1$_{12-20}$, Her2/neu$_{369-377}$, MAGE-2$_{112-120}$, PSA$_{141-149}$, BA46$_{97-105}$, cyclin D1 101-109, Hepa$_{183-191}$, bcl-2$_{218-226}$, and p53$_{149-157}$ (4, 13–22).

Generation of DC and TC. Dendritic cells (DC) were generated according to standard procedures from BM precursor cells by 14-d culture in serum-free medium with rhuGM-CSF and interleukin (IL)-4 as described (4). Afterwards, DC were enriched by depletion of contaminating TC and B cells (BC) and pulsed for 20 h with lysates (200 μg protein/1×10^6 cells/mL) from freshly isolated autologous tumor cells or PB mononuclear cells (PBMC), or from allogeneic cell lines as described (4), or loaded with 10 μg/mL HLA-A*0201–restricted peptides (5, 8) or with 200 μg/mL MUC1$_{tr(137-157)5}$ (7). To generate TC, BM and PB cells were incubated for 13 d in RPM1 1640 with
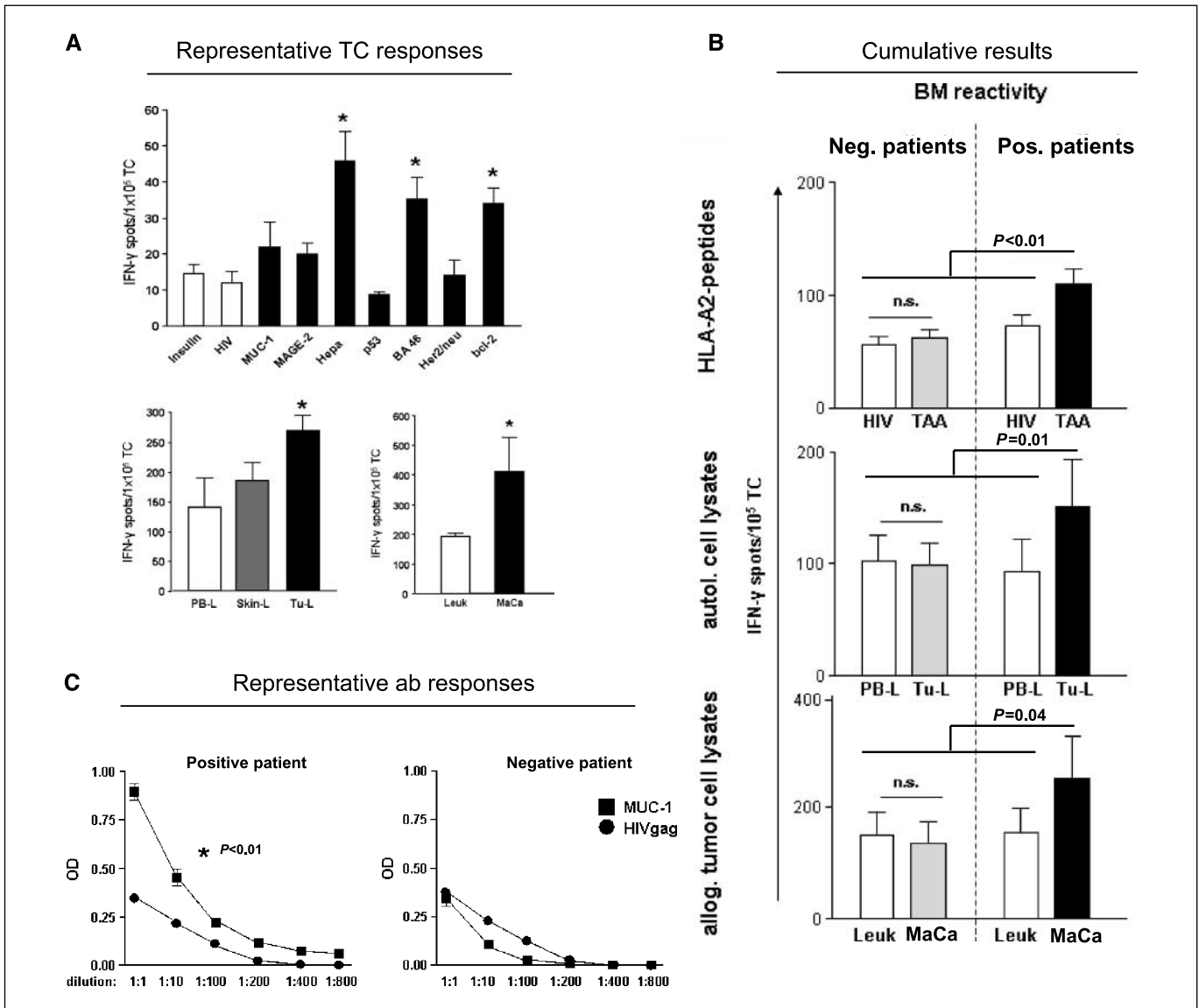
A Representative TC responses

B Cumulative results

Figure 1. Detection of tumor-specific immune responses. *A,* IFN-γ ELISPOT assays with BMTC from exemplary patients using as test antigens (*black columns*) HLA-A2–restricted tumor peptides (*top*), autologous tumor or skin lysate (*bottom left*), or allogeneic breast tumor lysate (*MaCa*; *bottom right*) compared with respective negative control antigens from HIV or insulin, autologous PBMC lysate (*PB-L*), or U937 lysate (*Leuk*; *white columns*). *Columns,* means of triplicate wells; *bars,* SEM. *, significant (*P* < 0.05) difference to negative control wells by two-sided Student's *t* test. *Hepa,* heparanase. *B,* mean IFN-γ spots in test and control wells of ELISPOT-positive and ELISPOT-negative patients. *TAA,* HLA-restricted tumor peptides; *TU-L,* autologous tumor lysate; *PB-L,* autologous PBMC-lysate; *Leuk,* allogeneic U937 leukemia lysate; *MaCa,* allogeneic MCF7 breast cancer cell lysate. *P,* statistical difference between test groups from positive patients and indicated control groups. *C,* representative ELISA results from two breast cancer patients demonstrating the presence (positive patient, *left*) or absence (negative patient, *right*) of MUC1-specific IgG. *OD,* absorbance; *, significant differences as determined by two-sided Student's *t* test.

10% human AB serum (PromoCell), IL-2 (100 U/mL; Chiron), and IL-4 (60 U/mL) followed by overnight culture without ILs. After depletion of $CD19^+$, $CD15^+$, and $CD56^+$ cells, the suspension contained 95% to 99% $CD3^+TC$ (~25% were $CD8^+$). In some experiments, cultured TCs were sorted for $CD45RO^-$ naïve cells using anti–CD45RO MACS beads (Miltenyi Biotec).

TC staining with HLA-A*0201-peptide tetramers. TCs were incubated for 10 min on ice with phycoerythrin-conjugated HLA-A*0201 pentamers with Her-2/neu–derived $peptide_{369-377}$ (Proimmune; ref. 4), and FITC-CD8 monoclonal antibodies (5). Recordings were made on propidium iodide–negative cells using FACScan and CELLQuest software (Becton Dickinson).

IFN-γ ELISPOT assays. IFN-γ–producing TC were determined as described (4). DC pulsed with different antigens were coincubated with autologous TC (DC/TC ratio, 1:5) for 40 h. IFN-γ-spot–forming cells were counted using a microscope Axioplan 2 and KS ELISPOT software (Carl

Zeiss Vision). Spots measured in the presence of DC pulsed with negative control antigens were considered nonspecific background. Individuals were designated as responders if the numbers of spots in the presence of DC loaded with TA were significantly higher (*P* < 0.05) than in negative control wells. The frequency of tumor-reactive TC was calculated as follows: (spot numbers in wells with TA-pulsed DC – spot numbers in negative control wells)/TC numbers per well.

Cytotoxicity assays. The ^{51}Cr-release assay was performed as described (4). Briefly, HLA-A*0201+ T2 cells were labeled with ^{51}Cr, pulsed with 10 μg/mL Her-2/$neu_{369-377}$ test peptide or HIVgag control peptide, and used as target cells. TC were cocultured with target cells for 4 h without further prestimulation.

Histopathologic evaluation of breast tumors. Tumors were classified with respect to the tumor-node-metastasis classification, and graded

according to Scarf-Bloom-Richardson. Evaluation of estrogen receptor (ER) expression was performed by immunohistochemistry according to the immune reactive score criteria. The status of receptor expression was considered negative when ER expressions scored <2.

Estimation of cancer-related mortality. The probability to die of breast cancer was estimated according to established prognostic factors using a Web-based algorithm (Adjuvant!)[8] recommended by the American Society of Clinical Oncology (23).

Cytokine quantification. Tumor and normal breast tissue was mechanically homogenized and processed as described (4). Cytokines were quantified using the multiplex protein array system technology (Bio-Rad Laboratories) or by respective HS Immunoassay Quantikine kits transforming growth factor (TGF)β1, IFN-α] according to the manufacturer's protocol (R&D Systems).

***In vitro* priming of tumor-specific TC.** DC were pulsed with 200 µg/mL autologous tumor lysate or $MUC1_{tr(137-157)5}$ together with 10 µg/mL rhuHsp 70 overnight. In some cases, DC were exposed to TGFβ1 (200 pg/mL) or IFN-α (20 pg/mL) during antigen uptake. After carefully washing DC and a 7-d coculture with naïve (CD45RO⁻) TC the presence of tumor-specific effector TC was evaluated using IFN-γ ELISPOT assay.

Detection of MUC1-specific antibodies. MUC1-specific antibodies were detected by ELISA as described (24). Briefly, 96-well plates were coated with 10 µg/mL $MUC1_{tr(137-157)5}$ or a control peptide HIVpol (ILKEPVHGV) and incubated with plasma samples at 1:1 to 1:800 dilution with PBS. Bound immunoglobulin was visualized using peroxidase-labeled anti-IgG or-IgM-antibodies (goat anti-human IgG/Fc-POX and goat anti-human IgM/Fc-POX, Jackson ImmunoResearch Laboratories) and ortho-phenylendiamine substrate (Sigma), and analyzed using an ELISA photometer. Samples were considered positive when triplicate test wells revealed a significantly enhanced extinction compared with triplicate control wells.

Generation of Her-2/neu–specific TC lines. Her-2/neu–specific CD8+ BMTC were isolated from HLA-A2⁺ patients using magnetically labeled $HLA-A*0201-Her-2/neu_{369-377}$ complexes (Tcap Dynabeads, Dynal) and expanded with anti CD3/CD28 beads (Dynal) according to the manufacturer's protocol. One week before testing, stimulator beads were removed and TC transferred into RPMI containing 100 U/mL IL-2 and 60 U/mL IL-4.

Statistical evaluation. Statistical analyses were done with two-sided Student's t test, χ^2 test, or Fisher's exact test. For visualization of multidimensional cytokine data and results from the IFN-γ ELISPOT assay, their correlation matrix (Pearson's correlation) was clustered by hierarchical clustering with the Euclidean distance metric and the complete linkage algorithm. The reordered matrix is presented as a color-coded heatmap. The clustering has been performed in R (R foundation for Statistical Computing, Vienna, Austria 2008. version 2.8.1).[9]

Results

Tumor-reactive TC in BM correlate with a reduced cancer mortality risk. From 181 of 207 study patients, clinicopathologic classification was obtained (Supplementary Table 1). None of the patients had received neoadjuvant treatment. BM was taken from 123 primary operated, nonmetastasized breast cancer patients during tumor resection. We determined the presence of functional tumor-reactive type-1 TC by IFN-γ ELISPOT assays. As test antigens, we used HLA-A*0201–restricted epitopes from breast tumor–associated antigens MUC1, Her2/neu, MAGE-2, prostate-specific antigen, BA46, cyclin D1, heparanase (Hepa), bcl-2 or p53 (4, 13–22), lysates from autologous breast tumor tissue (4), or lysate from the allogeneic breast tumor cell line MCF7 (4). Tumor cell–derived antigens efficiently restimulate a broad repertoire of preexisting, tumor-specific T-Helper and cytotoxic TC (25). Autologous DC loaded with respective antigens were used for restimu-

lation of *ex vivo* isolated BMTC during 40 h ELISPOT-assays. According to our previous findings within this short stimulation period, antigen-specific IFN-γ is exclusively secreted by preexisting memory TC (26). TC stimulated with irrelevant control antigens served for determination of the unspecific background. As control antigens served an HLA-A*0201–restricted epitope of HIV_{gag}, lysate of autologous PBMC, and lysate of the allogeneic leukemia cell line U937 (4). Exemplary IFN-γ ELISPOT data are shown in Fig. 1*A*. Antigen-specific responses, defined by a significantly increased spot number in triplicate test wells compared with corresponding control wells, are indicated by asterisks.

To verify that observed tumor-specific TC responses were not caused by unspecific decreases or increases of IFN-γ spots in control wells, we compared with total spots in responding and nonresponding patients. IFN-γ spots were similar in control and test wells of nonresponding patients, whereas test wells of responding patients showed significantly increased IFN-γ spots compared with the other groups (Fig. 1*B*).

TA-reactive TC were detected in 17 of 32 patients (53%) for HLA-A*0201–restricted peptides and highly individual with regard to antigen specificity. This is in accordance to a previous study (27) and might be due to individual differences in the expression rate of respective TAs. We detected TC reactivity against autologous tumor antigens in 19 of 58 patients (33%) and TC reactivity against allogeneic breast TAs in 19 of 33 patients (58%; Supplementary Fig. S1). In total, 55 of 123 patients (45%) showed the presence of tumor-reactive type-1 BMTC. TC reactivity against TAs was significantly increased in BM of patients compared with that of 19 healthy females either with regard to the proportions of responding individuals (45% versus 21%, $P = 0.03$) and to the proportions of positive test results (Supplementary Figs. S1 and S2).

Breast tumor–specific IgM and IgG responses were analyzed by ELISA, using synthetic peptide $MUC1_{tr(137-157)5}$ as test and HIV_{gag} as negative control antigen. Presence of MUC1-specific antibodies was assumed in case of significant differences in antibody binding between test and control triplicate wells. Representative results of patients with and without MUC1-specific IgG antibodies are shown in Fig. 1*C*. Thirty-one of 59 patients (53%) contained MUC1-specific IgG or MUCI-specific IgM antibodies (Supplementary Fig. S1). Ninety percent of them (28 of 59) were of IgM isotype, and 42% (13 of 59) were of IgG isotype. In contrast, we detected in sera of 11 healthy females no MUC1-specific IgG and MUC1-specific IgM in only 2 cases (18%, $P < 0.05$; Supplementary Fig. S1).

We used the prognosis algorithm ADJUVANT![8] (23) to compare antitumor immune responses with the estimated cancer-related mortality. Patients with tumor-reactive TC had a reduced mortality risk (HLA-A2 peptides, $P = 0.006$; tumor lysates, $P = 0.046$; Fig. 2*A* and *B*). In contrast, patients with MUC1-specific antibodies had the tendency for a higher risk of cancer-related mortality (Fig. 2*C, n.s.*).

Tumor-specific immune responses correlate with tumor pathobiology. We performed a subgroup analysis of clinicopathologic parameters to identify prognostic factors that correlated with spontaneous immune responses. TC reactivity was not correlated with the major prognostic factors, tumor size, lymph node involvement, or tumor stage (Fig. 3*A*), but instead with a high to moderate tumor cell differentiation, hormone receptor expression and low proliferative activity ($P = 0.05$, $P = 0.02$, $P = 0.05$, respectively; Fig. 3*B*). Because HER-2 is a prognostic factor in breast cancer and a target of tumor immune responses, we also evaluated a potential correlation of Her-2 expression with antitumor reactivity. In our study group, 24 among 176 patients were Her-2 positive and

Figure 2. Correlation of TC immunity and estimated cancer mortality. Reduced breast cancer mortality risk for patients with TC responses against tumor cell lysate (*A*, **+**, *light gray column*) or HLA-A*0201–restricted tumor peptides (*B*, **+**, *light gray column*) compared with TC-negative patients (*A* and *B*, **–**, *dark gray columns*). *C*, the mortality risk was not correlated with the presence (**+**; *light gray column*) or absence (**–**; *dark gray column*) of MUC1-specific antibodies. *Columns,* mean; *bars,* SEM. *, significant difference, *P* < 0.05 two-sided Student's *t* test. *n.s.,* not significant.
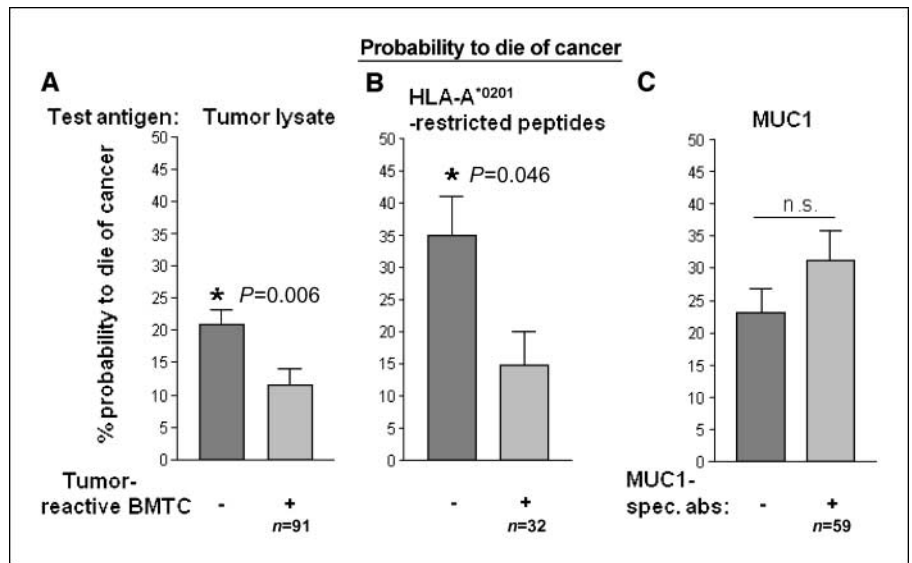
152 were Her-2 negative by immunohistochemistry and/or fluorescence *in situ* hybridization analysis (data not shown). We detected tumor-specific TC in 25% of patients with Her-2–positive but in 42% of Her-2–negative patients (not significant).

In contrast to TC responses, MUC1-specific antibodies correlated with increased tumor size and stage (*P* = 0.005 and *P* = 0.03, respectively, Fig. 3*A*) and were predominantly detectable in patients with lowly differentiated and receptor-negative tumors (not significant; Fig. 3*B*). These findings show that spontaneous tumor-specific TC responses correlate with a certain tumor cell phenotype rather than with tumor size or tumor cell presence in the lymphoid system. Accordingly, the rate of TC responses against HLA-A2–restricted tumor peptides or tumor lysate was strongly increased in patients with well-differentiated and ER-positive tumors (78% and 63%, respectively) when compared with patients with poorly differentiated, hormone receptor–negative tumors (16%, *P* = 0.05 and 5%, *P* = 0.004, respectively). In contrast, humoral responses were decreased in the former group of patients (19%, not significant; Fig. 3*C*). A majority of 70% of patients without TA-reactive TC showed instead TA-specific antibodies, whereas only few patients (21%) with a tumor-specific type-1 BMTC response contained TA-specific antibodies (*P* = 0.03; Fig. 3*D*).

Tumor pathobiology determines the functional capacity of TA-specific CD8+TC lines. The observed lack of TA-specific type-1 TC reactivity in patients with high-grade tumors could be explained by reduced numbers of TA-specific TC in these patients. Alternatively, such TC might have remained undetected in IFN-γ ELISPOT assay due to functional blockade. We therefore compared the numbers of Her-2/neu–specific CD8+TC by flowcytometry using Her-2/neu–loaded HLA-A*0201 tetramers. As shown in Fig. 4*A*, the frequencies of Her-2/neu–specific TC were increased in poorly differentiated tumors. Thus, reduced frequencies of TA-specific TC did not account for the observed lack of type-1 TC responses in patients with high-grade tumors. We therefore established Her-2/neu–specific CD8 TC lines from BM of 12 different HLA-A2+ breast cancer patients. TC lines were established by repeated isolation of antigen-specific cells with magnetic beads coupled to Her-2/neu-peptide–loaded HLA-A2 complexes (28) and subsequent polyclonal expansion of the cells. We used as antigen-presenting cells in subsequent functional assays T2 cells loaded with a defined high

amount of Her-2 peptide (20 μg/mL) to reduce the risk that insufficient TCR signaling (caused by different TCR avidities among the TC lines) accounted for putative functional differences. The TC lines were tested for their capacity to lyse Her-2/neu–loaded HLA-A2+ T2 target cells and/or to secrete IFN-γ upon specific stimulation. None of the TC lines were derived from patients with Her-2/neu overexpression. Figure 4*B* shows for one representative TC line the proportion of Her-2/neu–specific TC, its capacity to lyse Her-2–loaded target cells and to secrete IFN-γ in response to antigen-specific stimulation. In total, six TC lines showed functional capacity, whereas six were tolerant (Fig. 4*C*). Four of six lines from patients with low-grade tumors, and five of six lines from hormone receptor–positive patients showed functional capacity, whereas all six TC lines from patients with high-grade or hormone receptor–negative tumors were tolerant (*P* = 0.03 and *P* = 0.01, respectively; Fig. 4*D*).

Intratumoral cytokine patterns correlate with systemic tumor immune responses and tumor pathobiology. To assess potential reasons for the observed correlation of tumor pathobiology with systemic tumor-specific immune responses, we determined in lysates from 36 tumors the concentrations of 27 immune-modulatory cytokines, chemokines, and growth factors (Supplementary Table S2) by multiplex analysis. The results were statistically compared with the calculated frequencies of TA-reactive TC. These were calculated in positive samples by subtracting mean IFN-γ spots in negative control wells from mean spots in test wells. Figure 5*A* shows a heat map analysis of the cumulative results. Among all tested factors, only IFN-α was positively associated with increased TC responses, whereas intratumoral TGFβ1 was correlated with reduced TC frequencies. We also evaluated the presence or absence of tumor-reactive BMTC or MUC1-specific antibodies according to intratumoral expression of IFN-α and TGFβ1. Seventy-eight percent of patients with tumors containing TGFβ1 levels below the median concentration of 176 pg/mL together with detectable amounts (>0.1 pg/mL) of IFN-α, but only 21% of patients with increased intratumoral TGFβ1 and undetectable IFN-α showed the presence of tumor-reactive BMTC (*P* = 0.003; Fig. 5*B*). In contrast, MUC1-specific antibody responses were highly correlated with increased TGFβ1 and absence of IFN-α (*P* = 0.009; Supplementary Fig. S1; Fig. 5*B*). Accordingly, IFN-α was

significantly increased in tumors of patients with TC responses, whereas increased TGFβ1 correlated with reduced frequencies of tumor-reactive TC (Fig. 5C). Importantly, the concentrations of both cytokines in tumor tissue showed no correlation to the respective cytokine levels in corresponding PB samples (Supplementary Figs. S2 and S3). Thus, the local contents of TGFβ1 and IFN-α in breast tumor tissues were inversely correlated with the presence and frequencies of systemic tumor-reactive TC or tumor-specific antibodies.

IFN-α was increased in tumors of patients with well differentiated (G1/2) primary breast tumors ($P = 0.03$; Fig. 5D) compared with normal breast tissue and poorly differentiated (G3) breast tumors. In contrast, TGFβ1 was enhanced in poorly differentiated (G3) tumors ($P = 0.04$). Accordingly, the expression patterns of IFN-α and TGFβ1 in 51 primary breast tumors revealed two major subgroups containing either high levels of IFN-α together with low concentrations of TGFβ1, or the opposite pattern (Fig. 5D).

TGFβ1 and IFN-α influence priming of tumor-specific TC responses *in vitro*. We hypothesized that TGFβ1 and IFN-α in the tumor environment regulate systemic immunity by influences on immature DC (iDC) during antigen uptake. In this case, their respective concentrations should be sufficient to modify the capacity of iDC to prime type-1 TC responses. We therefore generated iDC from patients and pulsed them with autologous tumor lysate or with the synthetic long peptide MUC1$_{tr(137-157)5}$ (7) for 18 hours in the presence or absence of 20 pg/mL IFN-α or 200 pg/mL TGFβ1 at concentrations detectable in breast tumors. DC were then carefully washed and cocultured with separated autologous naïve (CD45RO$^-$) or memory (CD45RA$^-$) TC for 7 days. Priming of tumor-reactive TC was then evaluated by IFN-γ ELISPOT-assay. Representative results of two independent experiments are shown in Fig. 6A and B. In 3 of 11 cases, we detected an induction of tumor-reactive TC only when DC had been pretreated with IFN-α (Fig. 6A and C). Seven of 18 cultures spontaneously contained tumor-reactive effector TC (Fig. 6D). In six of these cases, TGFβ pretreatment inhibited the capacity of DC to prime tumor-reactive TC (Fig. 6B and D). DC-pretreatment with neither TGFβ1 nor IFN-α had no effect on memory TC of the same patients (data not shown). These findings indicate that IFN-α and TGFβ1 are present in breast carcinomas at sufficient amounts to regulate the capacity of iDC to induce primary TA-specific TC.

Discussion

We here describe a correlation between the type of systemic antitumor immune response and specific microenvironmental conditions at the primary tumor. Spontaneous tumor-specific
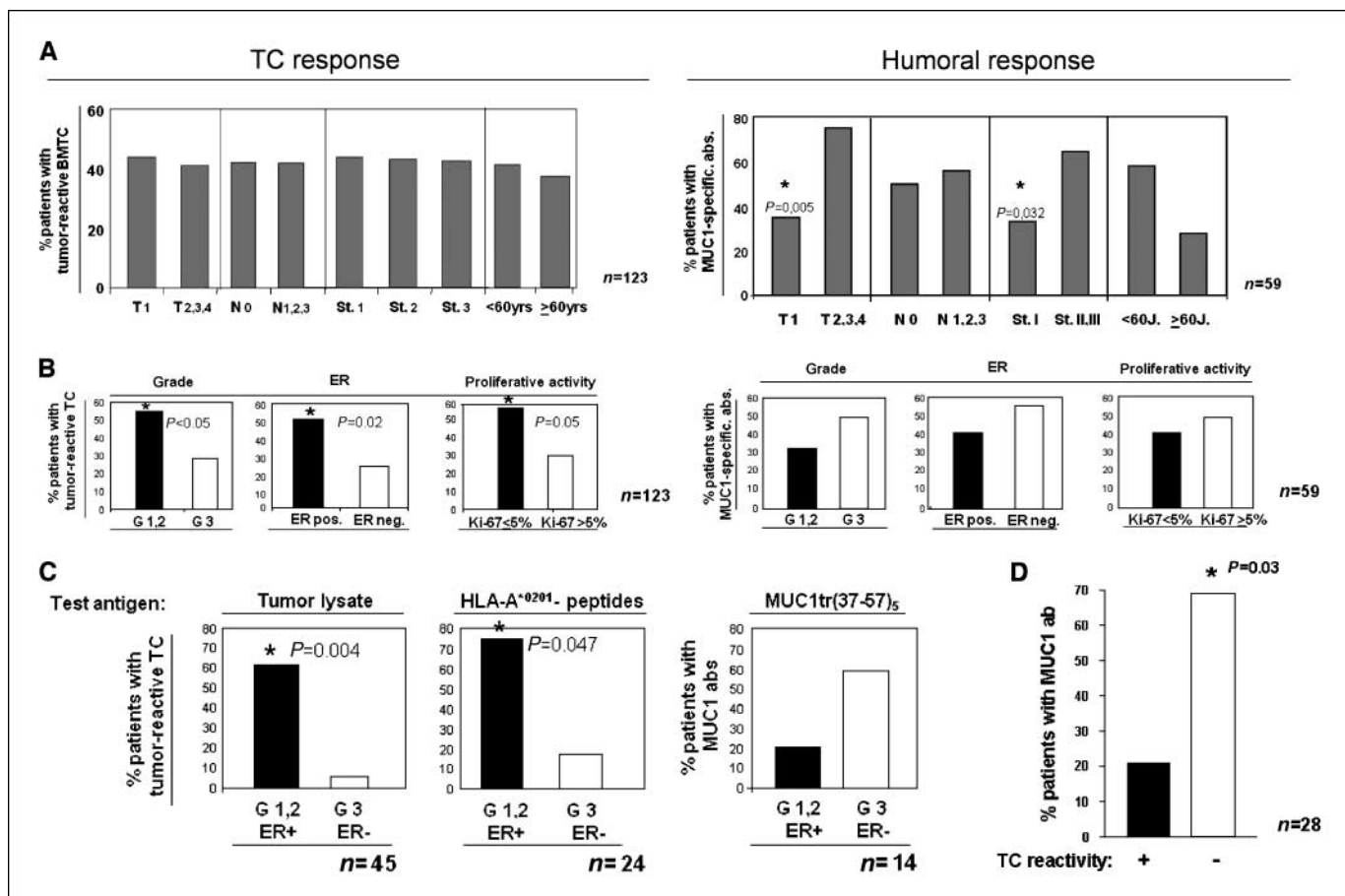
Figure 3. Antitumor immunity and clinicopathologic features. *A,* proportions of patients with tumor-specific TC (*left*) or antibodies (*right*) according to tumor size (*T*), lymph node involvement (*N*), stage (*St.*), and age (< or >60 y). *B* and *C,* correlation of TC responses with pathobiological features. Proportions of patients containing tumor-reactive TC (*left*) or antibodies (*right*) according to grade (*G*), ER expression, or proliferative activity (% Ki67+ tumor cells; *B*), or stratified into groups combining high tumor differentiation (*G1,2*) with ER expression (*ER*+) or low tumor differentiation (*G3*) and lack of ER expression (*ER*−; *C*). *D,* dichotomy of antitumor TC and antihumoral immune responses. Proportions of patients containing MUC1-specific antibodies according to the presence (+) or absence (−) of tumor-reactive TC. *, significant differences by χ² test (*A–C*) or Fisher's exact test (*D*); *n*, number of patients.
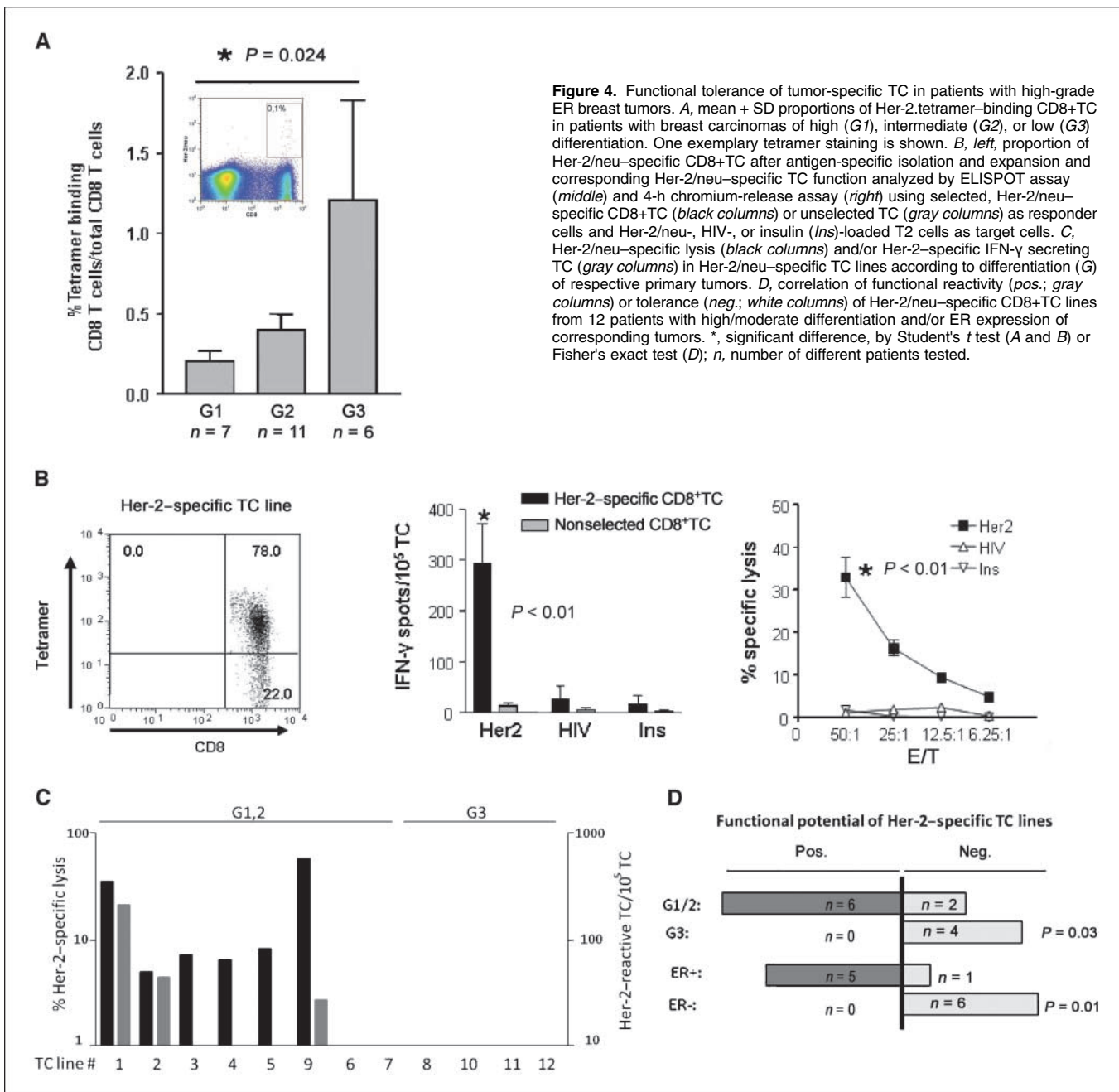
Figure 4. Functional tolerance of tumor-specific TC in patients with high-grade ER breast tumors. *A,* mean + SD proportions of Her-2.tetramer–binding CD8+TC in patients with breast carcinomas of high (*G1*), intermediate (*G2*), or low (*G3*) differentiation. One exemplary tetramer staining is shown. *B, left,* proportion of Her-2/neu–specific CD8+TC after antigen-specific isolation and expansion and corresponding Her-2/neu–specific TC function analyzed by ELISPOT assay (*middle*) and 4-h chromium-release assay (*right*) using selected, Her-2/neu–specific CD8+TC (*black columns*) or unselected TC (*gray columns*) as responder cells and Her-2/neu-, HIV-, or insulin (*Ins*)-loaded T2 cells as target cells. *C,* Her-2/neu–specific lysis (*black columns*) and/or Her-2–specific IFN-γ secreting TC (*gray columns*) in Her-2/neu–specific TC lines according to differentiation (*G*) of respective primary tumors. *D,* correlation of functional reactivity (*pos.; gray columns*) or tolerance (*neg.; white columns*) of Her-2/neu–specific CD8+TC lines from 12 patients with high/moderate differentiation and/or ER expression of corresponding tumors. *, significant difference, by Student's *t* test (*A* and *B*) or Fisher's exact test (*D*); *n*, number of different patients tested.

TC immunity was determined by the presence of IFN-α together with low concentrations of TGFβ1, whereas humoral immune responses were generated in the absence of intratumoral IFN-α together with increased TGFβ1. These findings suggest a role of both cytokines in the regulation of systemic antitumor immune responses. Because their concentrations in the blood were neither correlated with concentrations in the tumors nor with respective immune responses, it seems likely that the tumor tissue is a site where the induction of tumor-specific TC responses is regulated.

Indeed, the functional capacity of Her-2–specific BMTC depended on the tumors' pathobiology. Of note, we used in these assays peptide-pulsed T2 cells but not Her-2–positive breast cancer cells as TC targets. Therefore, the assays do not allow conclusions

regarding the potential clinical relevance of responding TC. A candidate population of cells that translate immunologic information from peripheral tissues to lymphoid organs are DC. In breast carcinomas, iDC are localized in the vicinity of tumor cells where they can take up antigen and may receive maturation stimuli (29). Antigen-pulsed DC migrate via lymphatics to draining lymph nodes but also via PB to the BM (30). The BM selectively recruits naïve and memory TC and antigen-pulsed DC to specialized microvascular domains. Here, activated DC induce tumor-specific TC responses (31, 32), whereas in the absence of activation stimuli during antigen uptake, DC become tolerogenic (33). Thus, microenvironmental conditions during antigen uptake control the function of resulting TC. Our data suggest that IFN-α and TGFβ1 may be involved in this process.
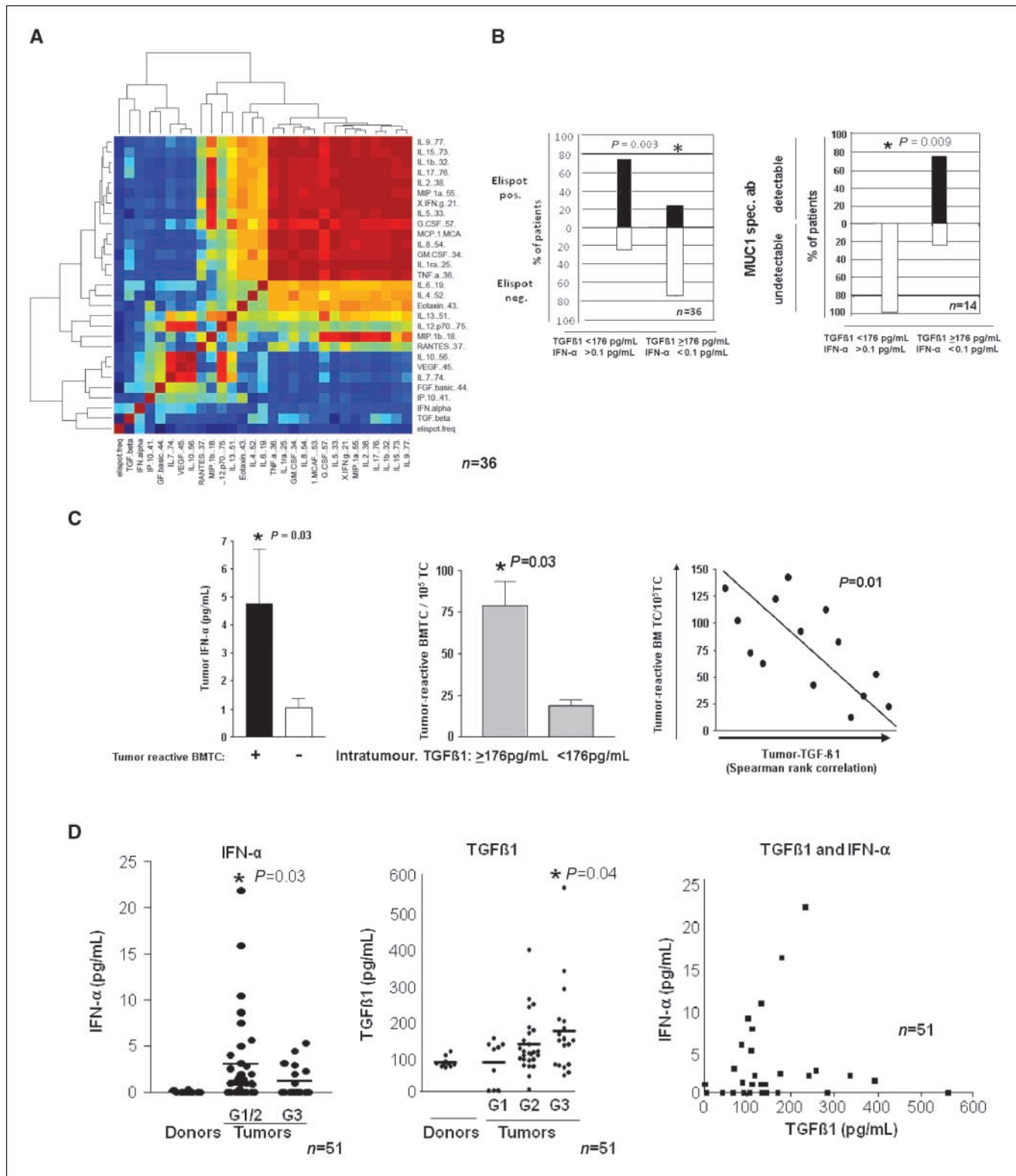
Figure 5. Correlation of systemic antitumor immune responses and cytokine content in breast carcinomas. *A,* heat map analysis of mean cytokine concentrations in 36 breast tumors and corresponding frequencies of tumor-reactive BMTC. *B,* proportion of patients containing (*black columns*) or lacking (*white columns*) tumor-reactive TC (*left*) or MUC1-specific antibodies (*right*) in patient groups characterized by TGFβ1 increased above the median value and undetectable IFN-α or reduced TGFβ1 and detectable IFN-α. *, significant difference (*left*, χ^2 test; *right*, Fisher's exact test). *C, left,* presence of tumor-reactive TC correlates with increased intratumoral IFN-α. *, significant difference (two-sided Student's *t* test). *Middle graph,* reduced frequencies of tumor-reactive TC correlate with increased TGFβ1 levels above the median of the whole group. *Columns,* mean frequencies; *bars,* SD. *, significant difference (χ^2 test). *Right graph,* Spearman's rank correlation demonstrating a significant inverse correlation between TGFβ1 contents in primary breast tumors and frequencies of tumor-reactive TC. *D,* concentrations of IFN-α (*left*), TGFβ1 (*middle*), or of IFN-α and TGFβ1 (*right*) in tumor lysates from 51 primary breast tumor tissues or in lysates of normal breast tissue (*donors*) determined by ELISA. *Dots,* different patients or donors. *, significant difference (two-sided Student's *t* test).
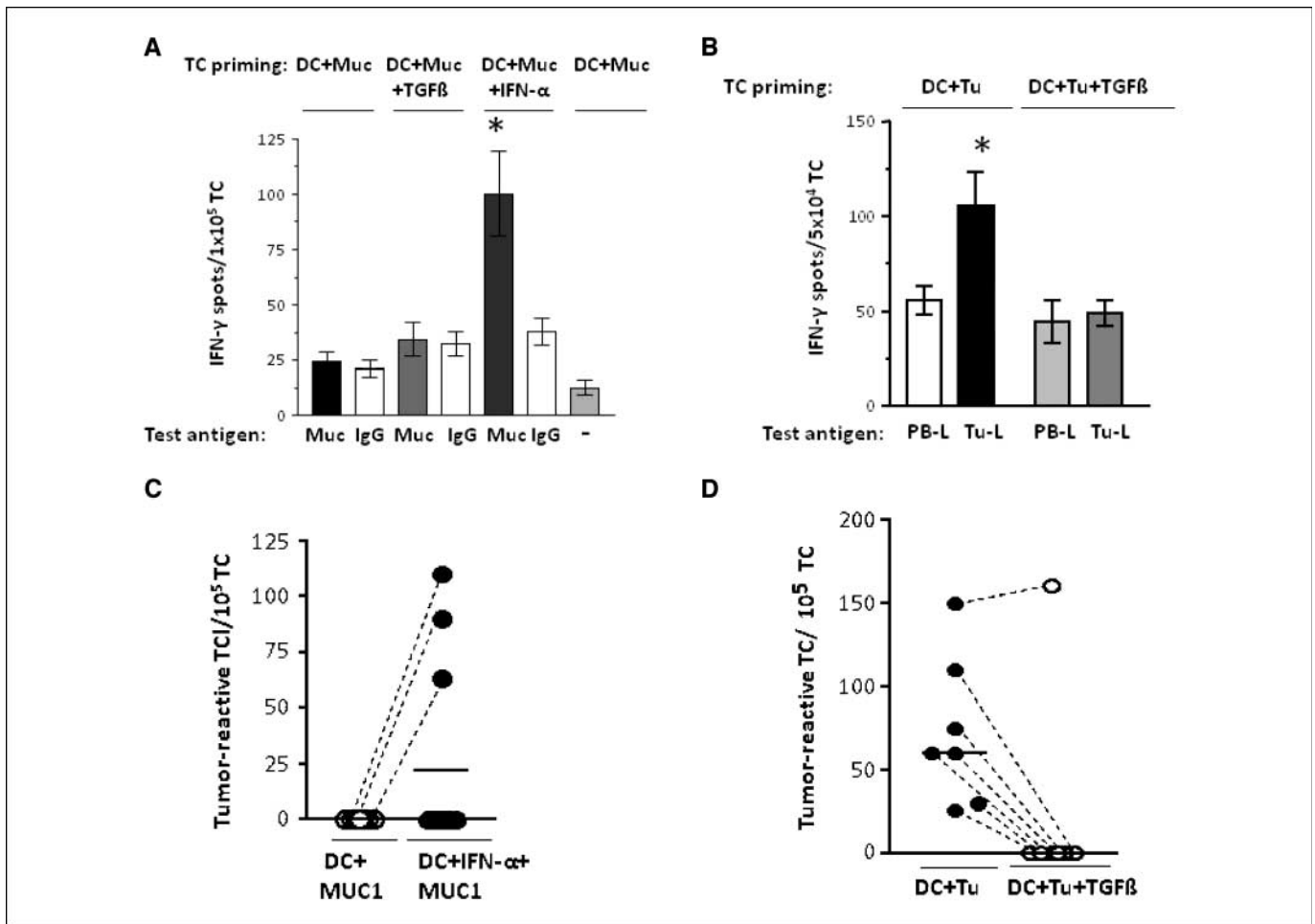
Figure 6. TGFβ1 and IFN-α influence priming of tumor-specific TC. Isolated naïve TC were stimulated for 7 d with TA-pulsed DC (*black columns*). In some cases, DC were coincubated during antigen uptake with 20 pg/mL IFN-α (*A and C*) or 200 pg/mL TGFβ1 (*B and D*). After 7 d, TCs were tested by IFN-γ ELISPOT assay for reactivity against TAs (MUC1$_{tr(137-157)5}$ or autologous tumor cell lysate (*black and dark gray columns*) or corresponding negative control antigens (huIgG or autologous PB-lysate; *white or light gray columns*). *A and B*, representative experiments show TC priming by pretreatment of DC with IFN-α (*A*) or abrogation of TC priming by DC pretreatment with TGFβ1 (*B*). *C*, cumulative frequencies of tumor-reactive TC induced by antigen-pulsed DC pretreated with IFN-α. *D*, cumulative frequencies of tumor-reactive TC induced by TA-pulsed DC pretreated with TGFβ1. *Dots*, samples of different patients. *Dashed lines*, connect samples from the same patients. *Horizontal full lines*, the mean frequencies of tumor-reactive TC. *, significant difference (two-sided Student's *t* test).

We showed that TA-pulsed iDC in the presence of IFN-α acquired the potential to prime tumor-specific type-1 TC even in the absence of further DC maturation signals. IFN-α is a major inducer of DC activation, DC homing to lymphoid organs, and efficient priming of type-1 CD8$^+$ and CD4$^+$ TC responses (34, 35).

In contrast, the presence of TGFβ1 during antigen uptake inhibited the capacity of iDC to prime tumor-specific type-1 TC *in vitro* (36). In tumors, TGFβ1 can be secreted by tumor cells, fibroblasts, regulatory TC, and macrophages. DC differentiated in the presence of TGF-β1 express low levels of MHC-II and of costimulatory molecules, consistent with an immature phenotype (36). Moreover, TGFβ1 inhibits DC maturation, antigen presentation, and the production of activation-induced mediators of inflammation (36).

TGFβ1 expression in tumors may also play a role in the observed correlation between tumor pathobiology and systemic immune responses. TGFβ1 activates stroma fibroblasts to secrete tumor-promoting growth factors (37). On the other hand, TGF-β induces cell cycle arrest and proapoptotic proteins (32). Therefore, the loss of TGFβ1 receptors (TGFβR) allows tumor cells to take advantage of tumorigenic TGFβ1 effects (35, 37). TGFβR loss occurs frequently in ER-negative tumors because ER-signaling maintains tumor cell differentiation and TGFβR expression (38). This may explain the correlation between low differentiation (caused by ER loss) and increased TGFβ1 contents in breast tumors in our study and may explain the increased mortality risk in patients lacking tumor-reactive TC.

Besides indirect influences on TC induction that are mediated by DC, it is conceivable that microenvironmental conditions in tumors directly affect systemic TC responses. Tumor-reactive TC can be recruited into tumors where they undergo functional inhibition that may be retained upon systemic recirculation.[10] Although TGFβ1 has minor effects on activated TC that is due to their reduced expression of TGFβR (36), it might be possible that TGFβ1 indirectly inhibits TC reactivation. TGFβ1 can trigger *in situ* the generation of immune-suppressive regulatory TC (39) that are associated in ER-positive breast tumors with reduced survival (40). Upon their recirculation from the tumor microenvironment into the lymphoid system, regulatory TC might inhibit the priming or reactivation of tumor-specific TC in lymphoid organs.

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[10] Our own unpublished observations.

In contrast to TC responses, MUC-1–specific humoral responses were correlated with increased tumor size and increased TGFβ1, which might explain their increased cancer mortality risk. Indeed, a previous study could only show a beneficial prognostic impact of MUC1-specific humoral responses in early breast cancer but not in the whole breast cancer population (41). Large tumors release higher amounts of soluble TA into BC areas of lymphoid organs and may thereby support *in situ* the activation of TA-specific BC. In the majority of cases, the observed antibody responses were of IgM isotype only, indicating a lack of further BC stimulation through TC. In other cases, observed IgG responses might have been supported by tumor-specific TH2 cells that were not analyzed in this study. Because we only evaluated spontaneous antibody responses against a synthetic peptide of MUC1 but not against the putatively more relevant, glycosylated endogeneous MUC1, we cannot exclude that antibodies specific for endogeneous MUC1 (42) or progression-relevant molecules exert protective effects even in advanced cancer patients. As spontaneous BC responses have been correlated with improved survival in a variety of other tumors, further analyses with a broader range of test antigens and immunoglobulin isotypes will be required to verify and extent our observations.

In conclusion, we here show the presence of two major subgroups of breast tumors that differ with regard to TGFβ1 and IFN-α content, tumor cell pathobiology, mortality risk, and TC immunity. Our findings suggest that TGFβ1 and IFN-α in the tumor microenvironment are involved in the regulation of systemic antitumor TC responses and may be promising targets of future individualized immunotherapy of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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