Stimulated γδ T Cells Increase the In Vivo Efficacy of Trastuzumab in HER-2⁺ Breast Cancer

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Aude-Hélène Capietto,*†,1 Ludovic Martinet,†,‡ and Jean-Jacques Fournié*†,§

One fourth of women with HER-2⁺ metastatic breast carcinoma are treated with a combination regimen with trastuzumab, but the frequent resistance to this Ab requires definition of new means to improve its bioactivity. The mechanisms of action of trastuzumab involve several pathways including Ab-dependent cellular cytotoxicity. Because human γδ T lymphocytes mediate Ab-dependent cellular cytotoxicity and can be activated further by phosphoantigens, these cells are prone to improve the efficacy of Abs, as recently demonstrated for CD20⁺ B cell lymphomas. Whether this concept applies as well with carcinomas remained to be demonstrated in vivo, however. In this study, we asked whether a combination of trastuzumab and phosphoantigen-stimulated γδ lymphocytes increases the efficacy of trastuzumab against HER-2⁺ breast carcinoma cell lines in vivo. We report that repeated infusions of this combination had a better efficacy than that of trastuzumab alone against HER-2⁺ mammary carcinoma xenografts in mice. In these models, reduction of tumor growth was observed together with trastuzumab opsonization of HER-2⁺ cells and tumor infiltration by γδ lymphocytes. In addition in humans, the mammary carcinomas of 27 of 30 patients showed significant γδ T cell infiltrates. Altogether, these findings indicate that combination of trastuzumab and stimulated γδ cells represents a new strategy to improve the efficacy of Herceptin (trastuzumab) in HER-2⁺ breast cancer. The Journal of Immunology, 2011, 187: 000–000.

A pproximatively 20–30% of human breast cancers over-express the human epidermal growth factor receptor 2 (HER-2/neu), most commonly due to amplification of the c-erbB-2 proto-oncogene (1). With one-half million new breast carcinoma occurrences diagnosed yearly in the United States and Europe, HER-2/neu positive tumors represent an important cohort. In breast carcinoma, overexpression of this receptor tyrosine kinase is associated with poor clinical outcome (1).

The humanized mAb trastuzumab (TTZ) directed against HER-2/neu (2) has become, when combined with chemotherapy, a standard of care for women with HER-2-overexpressing metastatic breast cancer. Several investigators have demonstrated that TTZ exerts additive or synergistic effects on tumor cell growth and has strong activity either as a single agent or in combination with cytotoxic chemotherapies (3, 4). This resulted in significant increases of overall response rate and survival time in addition to improved quality of life (5). However, a significant group of HER-2⁺ tumors remain primarily resistant or develop resistance to TTZ, although for still unknown reasons (6).

The in vivo mechanisms of action of TTZ are not completely understood, though their investigation in experimental models have suggested several possible mechanisms. These may comprise HER-2/neu downregulation (7), inhibition of cell cycle progression (8), impairment of signaling through other HER family members (9), impaired angiogenesis (10), and metastasis (11). Furthermore, there is evidence for induction of Ab-dependent cellular cytotoxicity (ADCC) by TTZ. Both preclinical and pilot clinical studies demonstrated that FcγR engagement on immune effector cells is a key mechanism of action for TTZ (12, 13). As several studies have demonstrated that TTZ resistance was neither associated with HER-2 downregulation nor with decreased ADCC (14, 15), enhancement of the TTZ-dependent immune responses represents a pertinent option. This view is supported by the following lines of evidence. First, inflammatory infiltrates are correlated with a better prognosis with HER-2-overexpressing breast cancer patients (16). Second, patients treated with TTZ plus chemotherapy or with TTZ followed by lapatinib significantly improved the clinical benefit, progression-free survival, and overall response (17, 18). Therefore, the stimulation of cell effectors of ADCC appears suited to increase the induction of this function by TTZ.

Human γδ T cells represent an important reservoir of lymphoid cell effectors for cancer immunotherapy. The majority of circulating γδ T lymphocytes in the adult (~1% of mononuclear cells) do not express CD4 or CD8 but harbor a Vγ9Vδ2 TCR specific for nonpeptide phosphoantigens (PAGs), a set of phosphorylated Ags from tumor cells and microbes (19, 20). Antigenic stimulation leads to TCR-Vγ9⁺ T cell activation, proliferation, and acquisition of effector functions with strong release of Th1 cytokines (e.g., IFN-γ, TNF-α) and cytolytic granules (21, 22). PAG-activated γδ T cells also mediate a strong cytolytic activity directed against cancer cells by direct (NK-like) cytolysis. Most Vγ9Vδ2⁺ T cells constitutively express the NKG2D coreceptor, which interacts
with tumors expressing MICA/B or ULBP1s contributes to cytolytic activation (23). Moreover, activated V9V82+ T effector memory cells upregulate the expression of CD16 (FcγRIIIA) to perform ADCC (24, 25).

Synthetic PAgS such as bromohydrin pyrophosphate (BrHPP) (26) and second-generation bioisosteres (27–29) have been developed as selective immunostimulating drugs targeting TCRVγ9V82+ T lymphocytes, most specifically for cancer immunotherapies. In addition, antioestrolytic drugs such as aminobisphosphonates activate the same lymphocytes by inducing endogenous PAgS and could thus have related therapeutic applications (30–32). These different molecules have led several groups to demonstrate anti-tumor functions of human γδ T lymphocytes in different in vivo contexts. Studies in nonhuman primates (33, 34) and in cancer patients (35–38) have illustrated the potential of activated TCRVγ9 T lymphocytes against leukemia, lymphoma, and carcinoma (reviewed in Refs. 39–42). Clinical trials involving PAgS or aminobisphosphonates alone and in combinations are currently being assessed as various anticancer therapies by different groups around the world. Along this line, several studies have shown the increase of lytic activity by γδ T cells combined with mAbs such as rituximab and TTZ in vitro (25, 43). In addition, clinical processes to amplify peripheral γδ T cells from cancer patients have been set up by several independent clinical laboratories (44–46), demonstrating the feasibility of γδ T cell-based cancer immunotherapies.

Nevertheless, whether transfer of γδ T cells combined with TTZ infiltrates human breast tumors and does better than TTZ alone remained untested. In this study, we show that TCRVγ9 T cells improve the bioactivity of TTZ in a murine model of human HER-2+ breast cancer xenograft. We find that with TTZ, TCRVγ9 T lymphocytes access the breast tumor site and are cytotoxic for HER-2-overexpressing breast tumor cells. Together with the demonstration of TCRVγ9 T lymphocytes infiltrating human breast tumors, this study indicates that combination of TTZ with γδ cell activation by PAgS represents a new strategy to improve the treatment of HER-2+ breast cancer.

Materials and Methods

Abs and flow cytometry

FITC-conjugated Abs to TCRV9, anti-CD4, PE–Cy5-conjugated anti-CD56, PE-conjugated Abs to markers TCRVγ9, CD3 or PE-Cy7-conjugated anti-CD45 were from Beckman-Coulter–Immunotech (Mar- seille, France), and Pacific blue-conjugated Abs to CD3 or CD8 and allophycocyanin–Cy7–conjugated anti-CD8 were from Ozyme (Saint Quentin en Yvelines, France). The respective isotype-matched conjugated controls were from Beckman-Coulter–Imunotechn and Ozyme, respectively. Flow cytometry was done with LSR-II and the dedicated software FACSDiva (BD Biosciences) and FlowJo 7.5.5 (Tree Star, Ashland, OR).

Reagents

The synthetic PAg BrHPP (Innate Pharma, Marseille, France) has been described elsewhere (26). Recombinant human IL-2 was kindly provided by Sanofi-Aventis (Toulouse, France) and TTZ (Herceptin; Roche, Basel, Switzerland) was a kind gift from Dr. Arnaud Cabelguenne (Institut Claudius Regaud, Toulouse, France). Cells were cultured in complete medium containing RPMI 1640 (Invitrogen, Cergy Pontoise, France) and TTZ (Herceptin; Roche, Basel, Switzerland). Human IL-2 (300 IU/ml at day 0, and further supplemented with recombinant human IL-2 (300 IU/ml) every 3 d but without further restimulation by BrHPP until injection in mice. In experiments involving mice treated four times, the injected TCRVγ9 T cells arose from the same healthy donor for all mice in each experiment. In the experiment with γδ T cell injections for 4.5 wk, TCRVγ9 T cells were expanded from four different healthy donors with each donor for two or more mice per group. The same scheme was used in both groups receiving γδ T cells. All experiments were done with V9V82+ T cell purity >95%.

Cell lines and cytotoxicity assays

The MDA-MB-231 and SK-BR-3 human breast cancer cell lines used as target cells were cultured at 37°C in complete medium with 10% FBS (Invitrogen). MDA-MB-231 was included as an HER-2/neu negative tumor cell line control, and SK-BR-3 was used to represent HER-2/neu positive tumor cells. Specific lysis by V9T cells was measured by standard 4-h 111In–Cr-release assays. The lysis rates were obtained by the following equation: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Maximum and spontaneous releases were determined, respectively, by adding 0.1% Triton X-100 or complete medium to 111In-labeled tumor target cells in the absence of γδ T cells. Data are presented as the mean of triplicate samples. Specific lysis assays by ADCC involved the same settings as above except the presence of TTZ or IgG1 (10 μg/ml) added to the 4-h cell cooincubation and to the spontaneous release and maximum release control experiments.

Tumor xenograft model

Eight- to ten-week-old female SCID/Beige mice were used to assess the in vivo anticancer properties of γδ T cells. SK-BR-3 cells (2 x 106) were suspended in PBS by trypsin and inoculated s.c. in the mammary tissue of mice. Tumor measurements were performed twice per week with a caliper, and volumes were calculated using the following formula: V = 1/2(length × width2). When tumors reached a mean volume of 40–150 mm3, the animals were randomized into groups with comparable tumor volume and treated. Treatments were injections of either γδ T cells, TTZ, combination of both, or vehicle control only. TTZ was given i.p. at a concentration of 10.0 mg/kg in sterile saline twice per week (vehicle control was saline) and 1 x 105 to 2 x 105 γδ T cells were administered i.v. (lateral tail vein) in sterile saline twice per week (vehicle control was saline). At the end of each experiment, tumors were removed at necropsy for FACS analysis. All animal protocols were submitted and approved by the local ethical committee under reference no. 311155505.

Flow cytometry of tumor xenografts and human tumor biopsies

Immediately upon sacrifice, xenografted tumors were excised then rinsed with PBS. Freshly excised tumors were then mechanically disaggregated, and cell suspensions were prepared, washed, and stained with Abs specific for human CD3 and TCRVγ9 and then analyzed by FACS.

Freshly resected human breast tumor samples or control samples of non-cancer breast tissues were reduced in small fragments and incubated 30 min at 37°C in sterile RPMI 1640 containing collagenase IV (1 mg/ml; Sigma-Aldrich, Lyon, France). Total cells were then extracted by mechanical dispersion and incubated 30 min at 4°C with Abs directed against different immune cell markers or their isotype-matched controls. Flow cytometry-based analyses were performed by FACS.

Statistics

One-tailed, paired Student t test was used whenever appropriate or one-way Mann–Whitney rank sum test was used otherwise using a threshold of α = 5% for significant differences (p < 0.05). All statistical analyses were performed using the Sigma Stat 3.0 (SPSS, Chicago, IL) and XL Stat 2008 (AddinSoft, Paris, France) software.

Results

In vitro cytotoxicity of V9V82 T cells with TTZ against HER-2+ cancer cell lines

V9V82 T cells are endowed with an NK-like HLA-unrestricted cytotoxic activity (47–49). Hence, to evaluate the in vitro cytotoxicity of V9V82 T cells with TTZ, the HER-2+ breast cancer cell line SK-BR-3 and the HER-2low breast cancer cell line MDA-MB-231 were used as allogeneic target cells (Fig. 1A). The γδ cell cytolytic activity was measured using the standard 51Cr-release assay and the detection of η6 T CELLS INCREASE THE EFFICACY OF TRASTUZUMAB...
on the in vivo growth of HER-2 high SK-BR-3 cells established as
lyzed the bioactivity of human
then tested the validity of this concept in vivo. To this aim, we ana-
On the basis of the above results from in vitro experiments, we
assay in six independent experiments with Vγ9Vδ2 T cells from
different healthy donors at various E:T ratios (Fig. 1B). The
addition of Vγ9Vδ2 T cells significantly increased the TTZ cyto-
toxicity (52.1 ± 8.6%, mean ± SEM) for SK-BR-3 compared
with TTZ alone (9.3 ± 1.3%, mean ± SEM, p < 0.05), Vγ9Vδ2 T
cells alone (11.0 ± 1.2%, mean ± SEM, p < 0.05), or Vγ9Vδ2 T
cells and human IgG control (10.3 ± 1.5%, mean ± SEM, p <
0.05) at an E:T ratio of 30:1 (Fig. 1C, left panel). With the HER-
2low targets MDA-MB-231 by contrast, the addition of
Vγ9Vδ2 T cells only had a marginal effect on TTZ cytotoxicity
(Fig. 1C, right panel), confirming that the cytolytic enhancement
observed with SK-BR-3 resulted from a better HER-2 targeting.
Thus, in vitro, TTZ combined with
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γδ T cells and TTZ inhibit mammary tumor growth in
SCID/Beige mice
On the basis of the above results from in vitro experiments, we
then tested the validity of this concept in vivo. To this aim, we ana-
alyzed the bioactivity of human γδ T cells combined with TTZ
on the in vivo growth of HER-2high SK-BR-3 cells established as
orthotopic breast cancer xenografts in mice. The SK-BR-3 cells
were implanted s.c. in the mammary tissue of SCID/Beige mice,
and without further manipulation they progressively grew as
tumors reaching ~140 mm³ within 60 d (Fig. 2A). Mice with these
established tumor xenografts were then treated twice per week for
2 wk with either vehicle control, TTZ alone (10 mg/kg), γδ T cells
alone (1 × 10⁷ cells), or both TTZ and γδ T cells.

Globally, the tumor growth in mice was more efficiently
inhibited by treatment with γδ T cells combined with TTZ than by
treatment with TTZ alone, γδ T cells alone, or vehicle control
(Fig. 2B). By 11 d of treatment, the tumor growth in mice that had
received γδ T cells alone or the vehicle control were nearly
similar: mean tumor growth was +50 mm³ with treatment by γδ
T cells alone versus +70 mm³ in the control group. At the same
time point, treatments by TTZ alone or by TTZ plus γδ cells were
equally effective: +28 mm³ in both groups. After 16 d of treat-
ment, however, TTZ plus γδ T cells (+12 mm³) did significantly
better than TTZ alone (+70 mm³) (p < 0.05, paired t test). Be-
cause the size of the tumor was significantly greater after 11 d of
TTZ alone but after 22 d with TTZ + γδ T cells treatment, the
bioactivity of the combination remained effective 11 d longer
despite the absence of further injections of the treatment (Fig. 2C).
Thus, a combination treatment with γδ T cells and TTZ inhibited
growth of an HER-2² breast carcinoma xenograft.

Because the previous experiments involved tumors challenged
while in exponential growth phase, however (day 56, Fig. 2A), the
same comparative treatments were performed at an earlier time
point of the tumor development, chosen in this study at day 41 of
the tumor engraftment (Fig. 2D). Growth of the tumor was mas-

γδ T cells infiltrate tumor xenografts and inhibit their growth
The above results raised the question whether increasing the
number of injections of γδ T cells and TTZ inhibited more effi-
ciently the tumor growth. To answer this, xenografted mice re-
ceived γδ T cells (2 × 10⁷ cells) and TTZ (10 mg/kg) injected
twice per week for 4 wk. The purity of primary γδ T cell lines was
checked at each time point before injection and was always >95%
(Fig. 3A). There was no tumor growth for 30 d in mice treated with
γδ T cells and TTZ together (37.0 ± 10.2 mm³ at day 0 versus
76.5 ± 48.3 mm³ at day 30, NS, paired t test). By contrast, tumor
grew significantly in mice treated with TTZ alone from day 18
(43.8 ± 9.0 mm³ at day 0 versus 177 ± 54.0 mm³ at day 18,
pair t test, p < 0.05) (Fig. 3B) and in mice injected with γδ T
cells alone or with the vehicle control (data not shown). The
better tumor growth in the “γδ T cell alone” group than in the
“trastuzumab alone” group was attributed to the proinflammatory
profile of these cells. As some mice from the “γδ T cells alone”
group were euthanized before the last injection, the mean of the tumor volume
was not determinable for the last time points (Fig. 3B, 3C). To check
whether γδ T cells were recruited to the tumor site, the
tumor xenografts were removed at necropsy and mechanically
disaggregated before staining with human anti-TCRVγδ and anti-
CD3 Abs. The rates of γδ tumor-infiltrating lymphocytes (TILs)
were then measured by flow cytometry. Infiltrating γδ T cells were
present in the tumor samples that had received TTZ and γδ T cells
but not in mice treated with TTZ alone. On the one hand, the rate
of γδ TILs was the same in mice treated with γδ T cells alone or
with γδ T cells plus TTZ (Fig. 3D). On the other hand, however,
tumors opsonized by TTZ were only present in mice treated with TTZ alone or with \( \gamma^d \) T cells plus TTZ (Fig. 3E).

These results indicated that in vivo, therapeutic regimens of \( \gamma^d \) T cells and TTZ combinations are able to do better than TTZ alone as they yield both tumor cell opsonization and tumor cell infiltration by the cytolytic \( \gamma^d \) T cells.

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**TCRV\(9^+\) T cells infiltrate human breast tumors**

To assess the clinical validity of this concept, the tumor biopsies of 30 patients diagnosed with breast tumor were analyzed for infiltrating immune cells. This comprised tumors collected after excision and histological tumor identification from 29 untreated patients with an infiltrative ductal carcinoma and 1 from a patient

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**FIGURE 2.** \( \gamma^d \) T cells and TTZ inhibit HER-2\(^+\) mammary breast tumor growth. A and D, In vivo growth of orthotopic mammary tumors (2 \( \times 10^6 \) SK-BR-3 cells) in SCID/Beige mice. Tumor volume of each group (\( n = 5 \)) before randomization and treatment (mean \( \pm \) SEM). B and E, In vivo growth of orthotopic mammary tumors (2 \( \times 10^6 \) SK-BR-3 cells) in SCID/Beige mice. Tumor volume of each group (\( n = 5 \)) after randomization and treatment (mean \( \pm \) SEM). Mice were treated four times with vehicle (\( \bullet \), untreated), TTZ (\( \bullet \), 10 mg/kg TTZ i.p.), \( \gamma^d \) T cells (\( \bullet \), \( 10^7 \) \( \gamma^d \) T cells i.v.), or TTZ and \( \gamma^d \) T cells (\( \bullet \), 10\(^7\) \( \gamma^d \) T cells i.v. + 10 mg/kg TTZ i.p.). C and F, Tumor volume of mice treated with TTZ (solid bars, 10 mg/kg TTZ) or with \( \gamma^d \) T cells and TTZ (open bars, 10\(^7\) \( \gamma^d \) T cells + 10 mg/kg TTZ). Mean \( \pm \) SEM. *\( p < 0.05. \)

**FIGURE 3.** \( \gamma^d \) T cells and TTZ infiltrate and inhibit HER-2\(^+\) mammary breast tumors. A, Primary \( \gamma^d \) T cell line purity used to inject at the different time points in mice (each week for 4 wk). Representative flow cytometric analysis from four different \( \gamma^d \) T cell lines used. Percentage of CD3\(^+\)TCRV\(9^+\) cells and isotypic controls (dashed lines) are shown. B, In vivo growth of orthotopic mammary tumors (2 \( \times 10^6 \) SK-BR-3 cells) in SCID/Beige mice. Tumor volume of each group (\( n = 7 \)) after randomization and treatment (mean \( \pm \) SEM). Mice were treated two times per week for 4.5 wk with \( \gamma^d \) T cells (\( \bullet \), \( 2 \times 10^7 \) \( \gamma^d \) T cells), with TTZ (gray bars, 10 mg/kg TTZ), or with \( \gamma^d \) T cells and TTZ (black bars, 2 \( \times 10^7 \) \( \gamma^d \) T cells + 10 mg/kg TTZ). Mean \( \pm \) SEM. *\( p < 0.05. \) ND, not determinable. D and E, Tumor biopsies from mice treated with \( \gamma^d \) T cells (\( 2 \times 10^7 \) \( \gamma^d \) T cells), with TTZ (10 mg/kg TTZ), or with \( \gamma^d \) T cells and trastuzumab (2 \( \times 10^7 \) \( \gamma^d \) T cells + 10 mg/kg TTZ) were removed and stained for flow cytometry analysis. Representative result from seven biopsies of each group. D, Tumor cells were stained with human anti-TCRV\(9\) and anti-CD3 Abs. Percentages of human \( \gamma^d \) T cells and isotypic controls (dashed lines) are shown. E, Tumor cells were stained with Fc-specific human IgG1 (Fc-specific hIgG1, solid line) or human IgG1 (hIgG1, dashed line) as control. Percentages of positive cells for TTZ stained on HER-2 are shown.
with an in situ ductal carcinoma. Twenty-three patients had positive expression for hormonal receptors, and four overexpressed HER-2/neu (Table I). We included in this analysis four control samples of noncancer breast biopsies to compare the proportion of TCRVγ9+ cells among other immune cells in nontumoral breast.

TCRγ9+ T cells were infiltrating all biopsies analyzed, although at variable rates. Only 3 of the 30 samples had barely detectable TCRVγ9+ cells, whereas 27 of the 30 harbored a clear-cut TCRVγ9+ lymphoid cell infiltrate (Fig. 4A). There were on average 150 γ6 lymphocytes for 100,000 total biopsy cells, almost as many NK cells (∼130 NK cells for 100,000 total biopsy cells), and much more CD4 and CD8 T cells (Fig. 4B). Quantifying the CD45+ TILs confirmed this bias: 5 and 8% of TILs from these ductal mammary carcinoma were TCRVγ9+ and NK cells, respectively, whereas the CD4+ and CD8+ subsets were prominent (50 and 37% of TILs, respectively) (Fig. 4C, 4D). In contrast, control samples of normal breast tissues consistently showed fewer TCRVγ9+ T cells than that of the above carcinomas. Furthermore, they also comprised fewer CD4 and CD8 T cells than that of the tumor samples, whereas NK cell numbers were in the same range in both groups (Fig. 4E–G). Altogether, these results demonstrated that TCRVγ9+ T cells infiltrate ductal mammary carcinomas from untreated patients.

### Discussion

Altogether, this study supported the view that the TCRVγ9+ T cells could be harnessed together with Herceptin to improve the efficacy of ADCC in the treatment of HER-2+ breast cancers. The treatment of HER-2/neu positive breast cancer has considerably benefited from the use of the therapeutic mAb TTZ. Frequent relapses, however, demonstrate that the bioactivity of this mAb is still suboptimal. Breast cancer and particularly metastatic breast cancer remain an important cause of mortality. If TTZ treatment improved the survival rate of patients with HER-2/neu positive breast tumor, many of them are or become resistant to TTZ. Nevertheless, several studies have demonstrated that the tumor resistance to TTZ is not the cause of an HER-2 expression decrease by cancer cells. Breast tumors that progress on TTZ continue to express high levels of HER-2/neu molecules (50, 51). Thus, approaches combining TTZ and other strategies to improve anticancer therapies remain to be developed.

Potentiating the ADCC induced by anticaner mAbs can be achieved not only by direct engineering of these drugs but also by strategies that target the downstream cytolytic effector cells (52).

### Table I. Patient characteristics

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<th>Parameter*</th>
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*Age of patients: mean ± SD, 58 ± 13 y; range, 30–85 y.

NK cells represent the major subset of lymphoid cells mediating ADCC. Patients induced to complete or partial remission by TTZ alone were found to have higher ADCC and higher infiltration of cytotoxic lymphocytes in situ (13). However, NK cell activities of PBMCs from breast cancer patients are significantly lower compared with those of healthy individuals (53), and there is a great heterogeneity of NK cell—ADCC activity among breast cancer patients. Moreover, assessment of ADCC in metastatic breast cancer patients treated with a combination of TTZ and IL-2 or with IL-12 alone failed to find that ADCC correlates with clinical responses (54, 55). Thus, transfer of immune cells stimulated to mediate a stronger ADCC represents an option to increase TTZ efficacy.

Among cell effectors of ADCC against tumor cells, a subpopulation of peripheral γ6Vδ2+ T cells expresses high level of FcγRIIIA and mediates cytotoxic function (22, 56). The recruitment of their FcγR-dependent functions appears well suited in this regard because FcγRIIIA-expressing γ6 T cells can be amplified, and the association of these cells with therapeutic mAbs improves their anticancer functions in vitro and in vivo (25, 43).

γ6 T cells have been consistently identified and isolated from the TIL population in various types of cancers, including breast carcinoma (57). In this study, we show that TCRVγ9+ T cells are present in biopsies of human mammary-infiltrating carcinomas with a similar proportion to NK cells, suggesting their attraction from peripheral blood to tumor site. In addition, the immune functions of TCRVγ9+ T lymphocytes against breast cancer cells suggest they contribute to the immunosurveillance of such cancers. Moreover, the absolute number and percentage of γ6 T cells from blood of cancer patients, including patients with bone metastasis of breast cancer, are the same as that in healthy donors (44). These γ6 T cells are cytotoxic against cancer cell lines after large-scale, good manufacturing practice-grade ex vivo expansion by PAg or zoledronate, and IL-2 is nowadays highly feasible (44).

All these observations suggested the development of novel alternative or adjuvant therapies using γ6Vδ2+ T cells and TTZ for treatment of HER-2/neu+tumor patients. The current work based on orthotopic human mammary carcinomas xenografted in SCID/Beige mice demonstrated that combining TTZ with γ6 T cells improves the in vivo efficacy of this Ab against an HER-2/neu+ breast cancer. Previous investigations have demonstrated the anti-tumor efficacy of human γ6 T cells in vivo after adoptive transfer into SCID mice inoculated with human tumor cells as various as lymphoma, nasopharyngeal carcinoma, and melanoma (58–60). Nevertheless, the presence of endogenous NK or macrophage murine cells could contribute to the antitumor activities observed in these studies. In addition, most primary cell lines of human Vγ9Vδ2+ T cells are biased toward a perforinlow Th1-type profile (21, 25). Hence alone, these cells might spontaneously kill mammary carcinoma very weakly (Figs. 1, 2), as this cytotoxic activity mainly relies upon release of perforin (25, 43). Furthermore, there is a vast body of literature indicating that proinflammatory lymphocytes and their corresponding cytokine profiles might actually promote growth of breast cancers (61, 62), as observed in this study in control groups of xenografted SCID/Beige mice receiving γ6 cells alone (Fig. 3B). Although future studies will determine the extent to which these observations translate into clinical practice, our results might help to interpret results from already initiated clinical trials involving autologous or allogeneic transplantation of primary cell lines of Vγ9Vδ2+ T lymphocytes alone.

Despite such limitations, however, this report showed that when human Vγ9Vδ2+ T cells were combined with mAb, these cells infiltrated the tumor and efficiently blocked its growth, confirming that ADCC is an important mechanism of action for Herceptin.
Thus, the use of this combined strategy needs not only to test ADCC and γδ-responsive patients but also a regimen that increases CD16 expression on these cells. The synthetic PAg BrHPP was shown to induce γδ T cell maturation toward terminally differentiated γδ T cells (TEMRA) and high-level CD16-expressing cells. This drug is nowadays available as good manufacturing practice-grade and is not toxic for patients. In addition, third-generation bisphosphonates indirectly stimulate Vγ9Vδ2+ T cells and thereby also increase their cytolytic activity.

Hence, the activity of this antiosteolytic, U.S. Food and Drug Administration-approved drug could also benefit this kind of combined therapeutic regimen. γδ T cell-based immunotherapy might be achieved for instance by stimulating Vγ9Vδ2+ T cells in patients through injection of PAg or bisphosphonate and IL-2, as recently performed in hematological malignancies (35), in prostate cancer (36), and in advanced metastatic breast cancer (64, 65). Nevertheless, it could be argued that such an approach could remain sensitive to tumor-induced immunosuppression. Most

FIGURE 4. TCRγ9+ T cells infiltrate human ductal mammary carcinomas. Total cells were extracted from freshly resected human breast samples, counted, stained, and analyzed by flow cytometry. A–D. Thirty breast tumor samples. A, TCRγ9+ T cell counts for 100,000 total biopsy cells. Median is shown (dashed line). B, Mean of cell counts for CD3⁺CD56⁺ cells (NK cells), CD3⁺CD8⁺ cells (CD8 T cells), CD3⁺CD4⁺ cells (CD4 T cells), and TCRγ9⁺CD3⁺ T cells (TCRγ9 T cells) for 100,000 total biopsy cells. Shown are mean ± SEM. *p < 0.05. C, Mean percentage of CD3⁺CD56⁺ cells (NK cells), CD3⁺CD8⁺ cells (CD8 T cells), CD3⁺CD4⁺ cells (CD4 T cells), and TCRγ9⁺CD3⁺ cells (TCRγ9 T cells) among CD45⁺ cells. Shown are mean ± SEM. *p < 0.05. D, Representative of TIL phenotypes (from patient no. 30). E–G, Four noncancer breast tissue (control) samples. E, Mean of cell counts for CD3⁺CD56⁺ cells (NK cells), CD3⁺CD8⁺ cells (CD8 T cells), CD3⁺CD4⁺ cells (CD4 T cells), and TCRγ9⁺CD3⁺ T cells (TCRγ9 T cells) for 100,000 total biopsy cells. Shown are mean ± SEM. *p < 0.05. F, Mean percentage of CD3⁺CD56⁺ cells (NK cells), CD3⁺CD8⁺ cells (CD8 T cells), CD3⁺CD4⁺ cells (CD4 T cells), and TCRγ9⁺CD3⁺ cells (TCRγ9 T cells) among CD45⁺ cells. Shown are mean ± SEM. *p < 0.05. G, Representative profile of lymphocytes infiltrating a nontumoral breast tissue sample.
notably, Vγ9Vδ2+ T cell proliferation and maturation could be inhibited by TGF-β (66), which is frequently released in the breast tumor microenvironment (67, 68). Even if in vitro, the strength of BrHP signaling can bypass TGF-β inhibition on Vγ9Vδ2+ T cells (66), we did not verify this in the orthotopic xenograft models depicted in this article. Moreover, other immunosuppressive cytokines and metabolites produced by tumors or their microenvironment might also regulate the γδ T cells, such as PGE2 (69–71). In addition, regulatory T cells might also dampen the antitumor function of γδ T cells (46). The alternative could be achieved by adoptive transfer of ex vivo-expanded autologous Vγ9Vδ2+ T cells from cancer patients (37, 38). The mice models assessed in this study were rather related to this approach, as they were treated with γδ T cell lines—albeit not autologous with SK-BR-3 cells—and amplified by in vitro culture with BrHP plus IL-2. Other options to restore full cytotoxicity against the tumor cells consist in additional administrations of zolodronate plus IL-2 (65), albeit repeated antigenic stimulation with PAg might finally produce a selective Vγ9Vδ2+ T cell energy in cancer patients (A.-H. Capietto, L. Martinet, and J.-J. Fournié, unpublished observations).

Nevertheless, this work indicated that the in vitro expansion of γδ T cells and their subsequent infusion with ITZ may be of significant clinical benefit in the treatment of HER2-neu positive breast cancers. Future clinical investigations from our and other laboratories will assess the therapeutic activity of these promising regimens.

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Disclosures
J.-J. Fournié holds an INSERM patent on therapeutic applications of phosphoantigens. The other authors have no financial conflicts of interest.

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