

## Antibody-Dependent Cell Cytotoxicity Synapses Form in Mice during Tumor-Specific Antibody Immunotherapy

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### Abstract

Antibody-dependent cell cytotoxicity (ADCC) plays a critical role in monoclonal antibody (mAb)-mediated cancer therapy. ADCC, however, has not been directly shown *in vivo* but inferred from the requirement for IgG Fc receptors (FcγR) in tumor rejection in mice. Here, we investigated the mechanism of action of a Tn antigen-specific chimeric mAb (Chi-Tn), which binds selectively to a wide variety of carcinomas, but not to normal tissues, in both humans and mice. Chi-Tn mAb showed no direct toxicity against carcinomas cell lines *in vitro* but induced the rejection of a murine breast tumor in 80% to 100% of immunocompetent mice, when associated with cyclophosphamide. Tumor rejection was abolished in Fc receptors-associated γ chain (FcR-γ)-deficient mice, suggesting a role for ADCC. Indeed, tumor cells formed stable conjugates *in vivo* with FcR-γ chain-expressing macrophages and neutrophils in Chi-Tn mAb-treated but not in control mAb-treated mice. The contact zone between tumor cells and ADCC effectors accumulated actin, FcγR and phospho-tyrosines. The *in vivo* formed ADCC synapses were organized in multifocal supra-molecular activation clusters. These results show that *in vivo* ADCC mediated by macrophages and neutrophils during tumor rejection by Chi-Tn mAb involves a novel type of multifocal immune synapse between effectors of innate immunity and tumor cells. *Cancer Res*; 71(15); 5134–43. ©2011 AACR.

### Introduction

The mechanisms of action of therapeutic monoclonal antibodies (mAb) are still matter of debate (1, 2). *In vitro*, certain therapeutic mAbs including trastuzumab (recognizing Her2/neu, used in breast cancers) or cetuximab (specific for the epidermal growth factor receptor, used in colon carcinomas) affect tumor cell growth by blocking receptor signalling. Other mAbs such as the anti-CD20 rituximab and the anti-CD52 alemtuzumab (both used in B cell neoplasia) induce apoptosis by direct transmembrane signaling (1, 3). In addition to these direct antitumor effects, therapeutic mAbs can kill tumor cells indirectly, through antibody-dependent cell cytotoxicity (ADCC), phagocytosis, or through complement dependent cell cytotoxicity (CDC). Although the mechanisms of tumor growth inhibition *in vivo* in animal models or in patients have

proved very difficult to unravel, there is good evidence that Fc receptors for IgG (FcγR) are somehow involved. This was shown for rituximab and trastuzumab-mediated immunotherapy in mouse tumor models (4–6) using different FcγR-deficient mice. Recently, de Haij and colleagues (6) showed that a mutation in the FcγR-associated γ (FcR-γ) chain that prevents cell activation and ADCC, but does not affect FcγR expression at the cell surface, also prevented antibody-mediated tumor rejection. In humans, patients bearing high affinity alleles of activating FcγRs (*FcγRIIIa*-158V/V and *FcγRIIIa*-131H/H genotypes) show increased clinical responses to rituximab or trastuzumab (7–9). Collectively, these data support a role for FcγRs and ADCC in the mechanism of action of therapeutic mAbs *in vivo*, although this phenomenon has not been directly shown.

The nature of the effector cell type responsible for the ADCC *in vivo* is also unclear. Macrophages and neutrophils are implicated in lymphoma rejection in mouse models using anti-CD20 mAbs (5, 10). The observed infiltration of breast cancers by natural killer (NK) cells in trastuzumab-treated patients suggests a role for this cell type in tumor rejection (11). In all published studies addressing the question, the immune system was reported to play a role in the therapeutic effect of the mAbs analyzed. Nevertheless, the antibodies used in the vast majority of these studies also have direct effects on the tumor cells, at least *in vitro*. Therefore, ADCC is required for tumor rejection, but we still do not know if it is sufficient.

We have investigated the potential use for immunotherapy of a mAb directed to the glycopeptidic antigen called Tn, using

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

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a mouse model of breast cancer. Tn is formed by a *N*-acetyl galactosamine residue (GalNAc) linked by *O*-glycosylation to serine or threonine amino acids present in the backbone of mucin-type glycoproteins (12). The Tn epitope (GalNAc-*O*-Serine/Threonine) is a cryptic determinant not detectable in normal cells, because it is masked by other sugar residues on mature saccharide chains. Aberrant protein glycosylation processes occur in almost 90% of human carcinomas (13–18), causing incomplete elongation of carbohydrate chains and unmasking the Tn antigen. Thus, Tn is one of the most widely expressed and specific tumor-associated antigens described so far, representing an attractive target for antibody-mediated passive immunotherapy (1, 2).

We now show that a chimeric anti-Tn mAb (Chi-Tn) recognizing a wide variety of epithelial cancers in both human and rodents (including breast and ovarian cancer, refs. 14, 19), has no direct effect on the growth or viability of the mouse breast cancer cell line TA3Ha *in vitro*. *In vivo*, however, Chi-Tn in association to cyclophosphamide (CTX) induced the rejection of TA3Ha tumors in more than 80% of mice. Tumor rejection *in vivo* required the FcR- $\gamma$  chain, indicating that the therapeutic effects observed depend on ADCC. Indeed, TA3Ha tumor cells interact *in vivo* with FcR- $\gamma$ + macrophages and neutrophils in Chi-Tn mAb-treated mice, forming an organized multifocal "ADCC synapse" at the contact zone.

## Materials and Methods

### Cells

TA3Ha (murine breast cancer, given by C. Leclerc, Institut Pasteur, Paris, France), TA3Ha-GFP (see Supplementary Methods), SKBR3 (human breast cancer, provided by P. de Cremoux, Institut Curie, Paris, France), Jurkat and RAW 264.7 cells were cultured in RPMI 1640 containing glutamax and 10% foetal calf serum. DOHH2 cells (human follicular B lymphoma) were purchased from DSMZ and cultured in RPMI 1640 containing 5% human AB serum.

### Mice

Female BALB/cByJ wild-type mice (7–8 weeks of age) were obtained from Charles River Laboratories. Fc $\gamma$ RIIb<sup>-/-</sup> [C.129S4(B6)-Fc $\gamma$ 2b<sup>tm1Tik</sup>/cAnNTac N12] and FcR gamma chain-deficient mice [FcR- $\gamma$ <sup>-/-</sup>, C.129P2(B6)-Fc $\gamma$ 1<sup>tm1Rav</sup> N12] were purchased from Taconic. Experimental procedures were approved by the French veterinary department.

### Antibodies

Rituximab (Mabthera, anti-CD20) and trastuzumab (Herceptin, anti-Her2) were purchased from Hoffmann-La Roche. The chimeric Chi-Tn mAb described in (20) was produced as explained in Supplementary Methods. These mAbs were biotinylated (NHS-LC-biotin, Pierce Protein Research Products) using manufacturer's instructions.

### Immunohistochemistry

Ovarian adenocarcinomas and breast cancers samples were obtained from patients of the Institut Curie, Paris, France. Tissues sections from paraffin-embedded tumor samples were

labelled with the biotinylated Chi-Tn mAb and avidin coupled to horseradish peroxidase.

### Flow cytometry

Cells were labelled using the indicated antibodies at 20  $\mu$ g/mL and with a F(ab')<sub>2</sub> goat antiserum specific for the human IgG Fc fragment (GaH-Fc) coupled to phycoerythrin (PE; GaH-Fc-PE; Jackson ImmunoResearch Laboratories), or with streptavidin-PE (Jackson). 4',6-diamidino-2-phenylindole (DAPI)-negative living cells (10<sup>4</sup>) were acquired using a fluorescence-activated cell sorting (FACS) Canto cytometer (BD Biosciences), and analyzed using the FlowJo software (Tree Star Inc.).

Ascitis samples (5  $\times$  10<sup>6</sup> cells) from ovarian cancer patients of the Institut Curie were saturated with human immunoglobulins, then labelled with anti-Epcam-FITC (fluorescein isothiocyanate), anti-CD45-PerCP-Cy5.5 (both from BD Biosciences) and biotinylated Chi-Tn mAb plus streptavidin-PE. Living cells (10<sup>6</sup>) were acquired.

For inhibition experiments, the Chi-Tn mAb (20  $\mu$ g/mL) was preincubated with synthetic GalNAc, or *N*-acetyl-D-glucosamine (GlcNAc) as control antigen (both from Sigma) at 1 mol/L final concentration for 1 hour at room temperature, then used for cell labelling as above.

For viability experiments, cells (10<sup>5</sup>) were plated in a 96-W round bottom plate in the presence of the indicated mAb for 15 minutes on ice. When indicated, unlabelled GaH-Fc (Jackson) was added at 100  $\mu$ g/mL final concentration to cross-link Chi-Tn. Cells were then transferred to 37°C for 16 hours, labelled with propidium iodide (PI), and the percentage of PI-positive cells was determined in the entire cell population (10<sup>4</sup> cells) by flow cytometry.

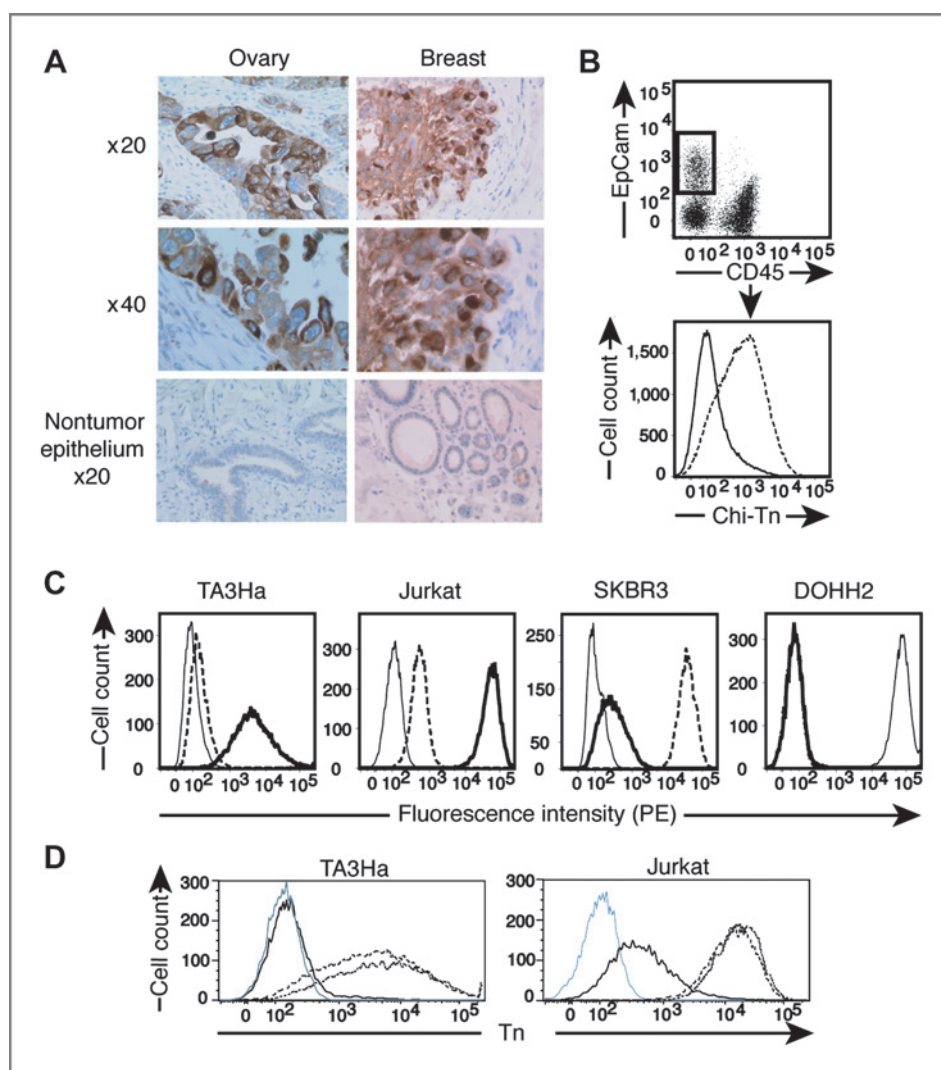
### Proliferation assays

SKBR3 (5  $\times$  10<sup>3</sup>) and TA3Ha cells (10<sup>4</sup>) were cultured in a 96-W flat bottom plate with Chi-Tn mAb or trastuzumab for 6 days at 37°C. Cell viability was then assessed by using the MTS assay (Promega). Results are expressed as a percentage of inhibition compared with untreated cells.

When indicated, cyclophosphamide (Sigma-Aldrich) or its active metabolite phosphoramid mustard (PhM, NSC69945, from the National Cancer Institute/NIH Developmental Therapeutics Program) were added to the cell culture. Cell viability was determined using the Alamar Blue assay (Promega) and results are expressed as arbitrary fluorescent units.

### *In vivo* tumor immunotherapy

Mice were injected intraperitoneally (i.p.) on day 0 with 10<sup>3</sup> TA3Ha tumor cells. The day after graft (day 1), mice were injected i.p. with CTX at 50 mg/kg or with PBS when indicated. Starting from day 2 after graft, mice were treated twice a week with the Chi-Tn mAb or the isotype control antibody (trastuzumab) at 20 mg/kg i.p. or with the same volume of PBS (6 total injections). Mice survival was monitored over a 2.5 months period of time; Kaplan–Meier survival curves and statistical analyses were done by using the log-rank test and the Prism 4 software (GraphPad Software).



**Figure 1.** Antigen-specific binding of the Chi-Tn mAb to the plasma membrane of ovarian and breast cancers. **A**, a representative human serous ovarian adenocarcinoma and a ductal infiltrating breast cancer were labelled with the biotinylated Chi-Tn mAb (brown areas). Magnifications  $\times 20$  and  $\times 40$  are shown. **B**, cells from a representative ovarian cancer ascites of patient were labelled with anti-Epcam-FITC, anti-CD45-PerCP-Cy5.5, biotinylated-Chi-Tn mAb (dashed line) or biotinylated-Chi-Tn mAb preincubated with GalNAc (continuous line), then with streptavidin-PE. Epcam + CD45-epithelial cells were gated in DAPI-negative living cells, and the Chi-Tn (PE) mean fluorescence intensity (MFI) was determined (MFI Chi-Tn + GalNAc: 239; MFI Chi-Tn: 1693). **C**, TA3Ha, Jurkat, and SKBR3 cells were incubated with rituximab (thin line), trastuzumab (dashed line) or Chi-Tn mAb (bold line), and GaH-Fc-PE. DOHH2 cells were labelled using biotinylated rituximab (thin line) or biotinylated trastuzumab (dashed line) or biotinylated Chi-Tn (bold line), and streptavidin-PE. **D**, Chi-Tn mAb was preincubated with soluble GalNAc (thin lines) or GlcNAc (control sugar, dashed lines). Jurkat and TA3Ha cells were incubated with each mixture or with the Chi-Tn mAb alone (dotted line), then with GaH-Fc-PE. Gray histograms: cells incubated with GaH-Fc-PE alone.

### ADCC assay

Gamma interferon ( $\text{IFN}\gamma$ )-primed RAW cells were plated at various effector-to-target (E/T) ratios with Chi-Tn mAb or trastuzumab (20  $\mu\text{g}/\text{mL}$  final concentration), and target TA3Ha cells labelled with carboxyfluorescein diacetate, succinimidyl ester (CFSE). After 24 hours at  $37^\circ\text{C}$ , cells were analyzed by flow cytometry according to (21) with some modifications (Supplementary Methods and Supplementary Fig. S1).

### Three-dimensional deconvolution microscopy

Peritoneal cells ( $10^6$ ) let to adhere to glass coverslips were fixed, permeabilized, labelled with the indicated primary antibody or with phalloidin-Alexa Fluor 546 (Molecular Probes, Invitrogen), then with the corresponding fluorochrome-conjugated secondary antibody. Cells were then stained with DAPI, and images were acquired using a three-dimensional (3D) microscope and deconvoluted (see Supplementary Methods).

## Results

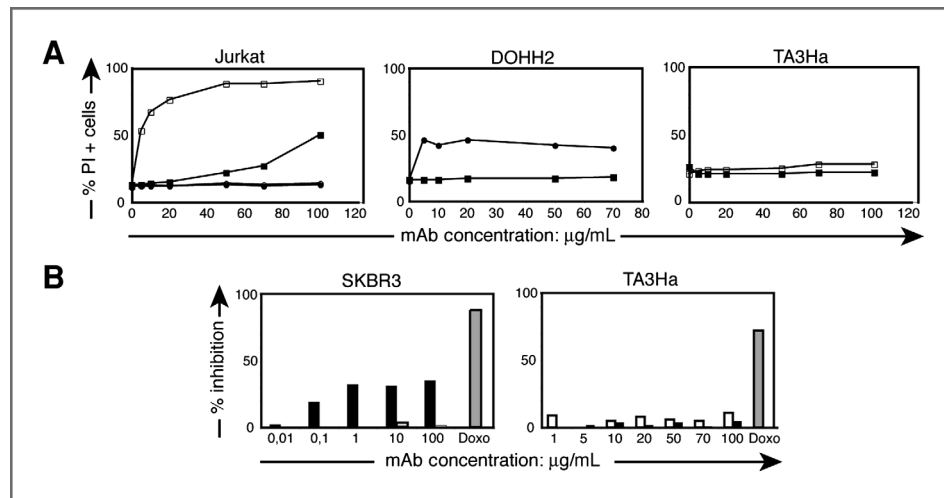
### The Chi-Tn mAb specifically labels human breast and ovarian cancers

The murine anti-Tn mAb (clone 83D4, IgM) recognized tumor cells from human carcinomas tissue sections but not the surrounding normal tissue (14, 15, 22). 83D4 strongly labelled more than 80% of ovarian and breast cancers (X. Sastre-Garau, unpublished results). Because the pentameric structure of IgM mAbs may impair penetration into the tumor, and because IgMs do not mediate ADCC, a chimeric mAb (Chi-Tn mAb) comprising the variable regions of the murine 83D4 mAb fused to the Fc portion of the human IgG1 was generated (20).

Figure 1A shows that, similarly to 83D4, the Chi-Tn mAb strongly labelled primary breast cancers and ovarian serous adenocarcinoma from patients. More than 80% of ovarian and breast tumor cells were labelled by the Chi-Tn mAb (top panel). Tn labelling appeared located in the cytoplasm and at



**Figure 2.** The Chi-Tn mAb does not affect TA3Ha cell viability or growth *in vitro*. A, DOHH2, Jurkat, and TA3Ha cells were cultured for 24 hours with Chi-Tn mAb (squares) or rituximab (circles), in the presence (open symbols) or absence (filled symbols) of GaH-Fc antiserum. Percentages of PI-positive dead cells were determined by flow cytometry. B, SKBR3 or TA3Ha cells were incubated with trastuzumab (black bars) or Chi-Tn mAb (white bars), or doxorubicin (positive control, 1  $\mu\text{g/mL}$ , gray bar) for 6 days. Cell proliferation was expressed as a percentage of inhibition compared with untreated cells.



the plasma membrane in both tumors (middle panel). Neither the healthy ovarian nor breast tissues were labelled with the Chi-Tn mAb (lower panel).

#### The Chi-Tn mAb specifically recognizes the Tn antigen at the plasma membrane of tumor cells

To determine whether the Chi-Tn mAb could be used in tumor immunotherapy, we analyzed the plasma membrane expression of the epitope in primary tumor cells from ovarian cancer ascites of patients. In 17 of 23 patients examined (74%), the Chi-Tn mAb labelled the ovarian tumor cells (Fig. 1B and Supplementary Table S1). The labelling was specific for the Tn antigen, since it was inhibited by soluble GalNAc (Fig. 1B). The Chi-Tn mAb strongly labelled Jurkat (human T lymphoma) and TA3Ha (mouse breast cancer) cells, whereas SKBR3 (Her2/neu-positive human breast cancer) and DOHH2 (CD20-positive human B lymphoma) cells were not recognized (Fig. 1C). Chi-Tn mAb binding to Jurkat and TA3Ha cells is shown in Supplementary Figure S2. Similarly, Chi-Tn mAb binding to these tumor cell lines was inhibited by GalNAc, but not by the control sugar GlcNAc (Fig. 1D). Therefore, Tn is expressed and accessible at the plasma membrane of fresh human ovarian cancer cells and of certain human and mouse tumor cell lines, which can thus be used as model systems to test the antitumor efficacy of this antibody.

#### The Chi-Tn mAb does not affect the viability or proliferation of TA3Ha cells *in vitro*

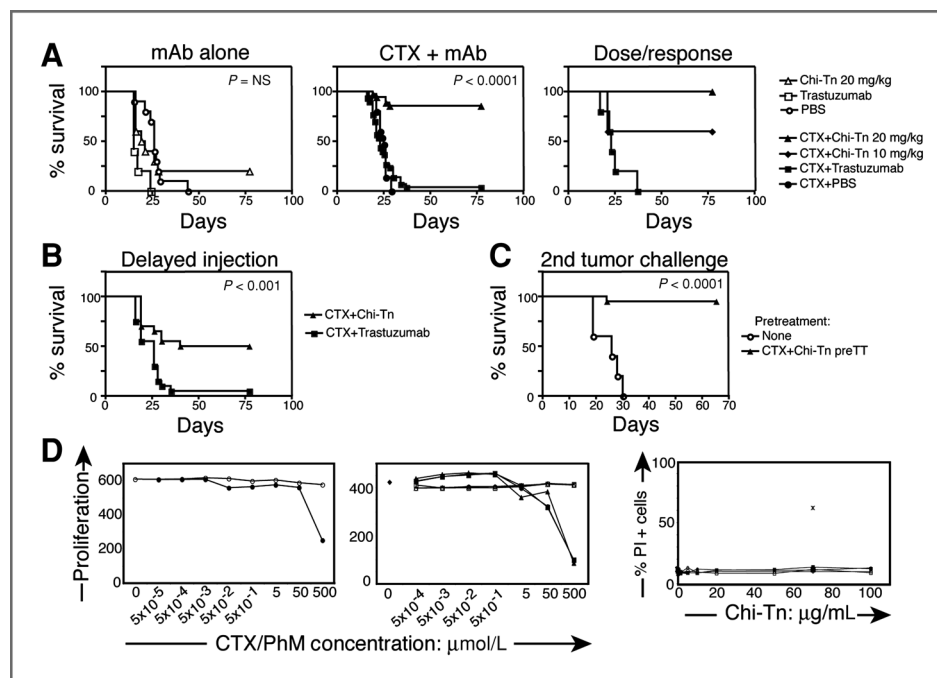
Most tumor cell-specific therapeutic mAbs used in the clinics, including trastuzumab, cetuximab, or rituximab, have a direct apoptotic or antiproliferative effects on tumor cell lines *in vitro*. These direct effects could account, partially or totally, for their therapeutic efficacy (1, 2). We thus determined whether the Chi-Tn mAb directly affects tumor cell viability *in vitro*. As shown in Figure 2A (left panel), moderate cell cytotoxicity (20%–40% of PI<sup>+</sup> dead cells) was observed when Jurkat cells were treated with high concentrations of Chi-Tn (50–100  $\mu\text{g/mL}$ ). Cross-linking the Chi-Tn mAb with a

secondary GaH-Fc antibody strongly increased Jurkat cell death (5  $\mu\text{g/mL}$  of Chi-Tn inducing 50% of PI<sup>+</sup> cells), most likely by increasing its avidity for Tn (Supplementary Fig. S3). As expected, the Tn-negative/CD20-positive DOHH2 cell line was not killed by the Chi-Tn mAb used at any concentration, although it was killed by the anti-CD20 rituximab (about 40% of cell death, Fig. 2A, middle panel). In contrast, direct tumor cell death was not observed for the Tn-expressing breast tumor cell line TA3Ha using either the Chi-Tn mAb alone or after cross-linking (Fig. 2A, right panel). The Chi-Tn mAb did not inhibit TA3Ha cell proliferation, while trastuzumab induced a 40% inhibition of SKBR3 cell growth (23, Fig. 2B). Similarly, cross-linked Chi-Tn mAb did not inhibit the proliferation of the Tn-positive human tumor cell lines MCF-7 and SHIN-3 (Supplementary Fig. S4). Thus, the Chi-Tn mAb did not display any direct apoptotic, or antiproliferative effect on Tn<sup>+</sup> epithelial tumor cells *in vitro*.

#### The Chi-Tn mAb associated with CTX inhibits TA3Ha cell growth *in vivo*

The efficiency of the Chi-Tn mAb at inhibiting tumor growth *in vivo* was assessed in the model of immunocompetent BALB/c mice grafted in the peritoneal cavity with TA3Ha mouse breast cancer cells. Control mice injected with trastuzumab or PBS rapidly developed ascitis (mean around day 11 in both cases), and then died (mean survival, 24 days; range, 15–26 days; Fig. 3A, left panel). Mice treated with the Chi-Tn mAb did not survive better than control mice (20% survival,  $P > 0.05$ ). No sign of general toxicity was observed in tumor-free mice injected with the Chi-Tn mAb alone (no loss of weight, abnormal activity or macroscopic organ lesion, data not shown).

Synergistic antitumor effects were observed previously when certain chemotherapies were combined with mAb treatment (24, 25). Moreover, low doses of CTX potentiated antitumor immune responses induced by immunotherapeutic treatments in mice, when CTX was given before vaccine injection (26, 27). Interestingly, as shown in Figure 3A (middle



**Figure 3.** Inhibition of TA3Ha cell growth *in vivo* after CTX + Chi-Tn mAb treatment. A, left: BALB/c mice grafted with TA3Ha cells (day 0), were treated from day 2 with Chi-Tn mAb ( $n = 10$ , open triangles) or trastuzumab ( $n = 5$ , open squares) at 20 mg/kg (6 total injections), or with PBS ( $n = 10$ , open circles). Middle: BALB/c mice grafted with TA3Ha cells were injected with CTX (50 mg/kg, day 1 after graft), then treated (6 injections) with the Chi-Tn mAb ( $n = 35$ , filled triangles) or trastuzumab ( $n = 30$ , filled squares), or PBS ( $n = 15$ , filled circles). Right: BALB/c mice ( $n = 5/\text{group}$ ) were given TA3Ha cells on day 0, CTX on day 1, then treated with Chi-Tn mAb at 10 mg/kg (filled diamonds) or at 20 mg/kg (filled triangles), or with trastuzumab at 20 mg/kg (filled squares) for 6 injections. B, BALB/c mice were grafted with TA3Ha (day 0) and injected with CTX (day 4). Chi-Tn mAb (filled triangles) or trastuzumab (filled squares) treatment started from day 5 at 20 mg/kg (6 injections). C, BALB/c mice which survived for at least 75 days after CTX + Chi-Tn treatment (CTX + Chi-Tn pretreated mice,  $n = 19$ , filled triangles) and naive mice not grafted and not treated before ( $n = 5$ , open circles) were rechallenged with  $10^3$  TA3Ha cells on day 0. Survival was then monitored. D, left: TA3Ha cells were cultured for 24 hours with various concentrations of CTX (open circles) or PhM (filled circles). Cell proliferation is expressed as arbitrary fluorescence units  $\times 10^{-3}$ . Middle: TA3Ha cells were cultured for 6 days with trastuzumab (triangles) or Chi-Tn mAb (squares) at 20  $\mu\text{g/mL}$  in the presence of graded amounts of CTX (open symbols) or PhM (filled symbols). Cell proliferation was determined as above. Filled diamond, TA3Ha cells cultured in medium alone. Right: TA3Ha cells were precultured for 24 hours with CTX (open symbols) or PhM (filled symbols) at 0.5  $\mu\text{mol/L}$ . After washing, cells were cultured for 24 hours with Chi-Tn mAb (squares) or trastuzumab (circles), in the presence of GaH-Fc antiserum. As a control, Jurkat cells were incubated with Chi-Tn at 70  $\mu\text{g/mL}$  (star). Percentages of PI+ dead cells were determined by FACS.

panel), when mice were given a single injection of CTX (50 mg/kg) on day 1 after graft and prior Chi-Tn mAb treatment, their overall survival was highly increased (86% survival,  $P = 1.8 \times 10^{-13}$ ) compared with mice treated with CTX + trastuzumab (as an isotype control, 4% survival). Importantly, survival of mice treated with CTX alone or CTX + trastuzumab was identical to that of mice treated with PBS or trastuzumab alone ( $P > 0.05$ ), showing that CTX by itself did not affect TA3Ha growth *in vivo* (Fig. 3A, compare left and middle panels).

The antitumor effects of the Chi-Tn mAb *in vivo* was dose dependant. Indeed, survival was decreased when mice were treated with CTX and a lower dose of Chi-Tn mAb (60% and 100% survival for 10 and 20 mg/kg of Chi-Tn mAb, respectively; Fig. 3A, right panel). Moreover, when the CTX + Chi-Tn mAb treatment was delayed (CTX injected on day 4 and Chi-Tn mAb injections starting from day 5), the survival of treated mice was still increased compared with control mice (Fig. 3B,  $P = 0.0007$ ). In addition, when mice previously treated and cured by CTX + Chi-Tn mAb treatment were rechallenged

with TA3Ha cells i.p. after 75 days, more than 90% of the mice were resistant to this second graft (Fig. 3C), most likely due to an adaptative antitumor memory response. Taken together, these results show that the Chi-Tn mAb prevented the growth of TA3Ha cells when associated to CTX, in the absence of any apparent sign of toxicity.

To investigate a possible direct toxic effect of the CTX + Chi-Tn association on TA3Ha cells, we used the CTX liver-active metabolite, PhM. A single injection of CTX at 20 to 60 mg/kg to rodents or humans induced a peak of 20 to 30  $\mu\text{mol/L}$  PhM in the plasma after 30 minutes to 1 hour, which then decreased rapidly (PhM half-life about 50 minutes, refs. 28, 29). PhM was not directly toxic for TA3Ha cells *in vitro* for concentrations up to 50  $\mu\text{mol/L}$  (Fig. 3D, left panel). Moreover (Fig. 3D, middle panel), no synergistic antiproliferative effect was found when TA3Ha cells were cultured with Chi-Tn and PhM (concentration up to 50  $\mu\text{mol/L}$ ). In addition, when TA3Ha cells, precultured with PhM (0.5  $\mu\text{mol/L}$ ; or 30  $\mu\text{mol/L}$ , not shown), were stimulated using Chi-Tn mAb cross-linked with a GaH-Fc antibody, no cell death was observed

(Fig. 3D, right panel). Similar results were obtained when TA3Ha cells were cultured simultaneously with CTX or PhM, Chi-Tn and GaH-Fc (not shown). Finally, PhM did not sensitize TA3Ha cell death by Chi-Tn-mediated ADCC *in vitro* (Supplementary Fig. S5). These results show that Chi-Tn, even upon cross-linking and in the presence of CTX, has no toxic effects on TA3Ha cells, suggesting that the tumor inhibition observed *in vivo* is not due to a direct effect of the Chi-Tn mAb on tumor cells.

#### The Chi-Tn mAb kills TA3Ha cells by ADCC *in vitro*, and rejects TA3Ha cells through activating FcγR *in vivo*

Because the Chi-Tn mAb did not inhibit directly the growth of TA3Ha cells *in vitro*, we investigated if it could induce tumor cell death by ADCC (Ref. 21, and Supplementary Fig. S1). Figure 4A shows that the Chi-Tn mAb, but not an isotype control, induced the lysis of TA3Ha cells incubated with RAW (a macrophage cell line) as effector cells. Therefore, the Chi-Tn mAb mediates ADCC *in vitro*.

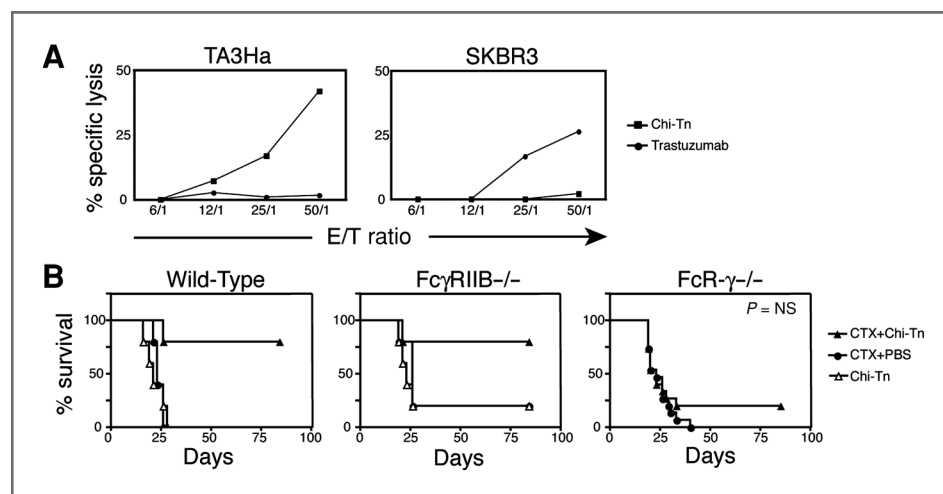
FcγRs were showed to play a role in the antitumor effect induced by different therapeutic mAbs in several mouse models of cancer (4, 5). We therefore examined whether these receptors were involved in the protective antitumor effect mediated by CTX + Chi-Tn mAb *in vivo*. The inhibitory FcγRIIB enhanced the antitumor effect of trastuzumab and rituximab in murine xenograft tumor models (4). In the present experimental model, treatment with CTX + Chi-Tn mAb induced the same level of protection in both TA3Ha-grafted FcγRIIB<sup>-/-</sup> and wild-type BALB/c mice (80% survival in each case,  $P = 0.937$ , compare Fig. 4B, left and middle panels). In addition, FcγRIIB<sup>-/-</sup> mice-bearing TA3Ha cells did not survive better than wild-type mice upon treatment with Chi-Tn mAb alone (20% and 0% survival respectively,  $P = 0.537$ , compare Fig. 4B, left and middle panels), and their survival was similar to CTX + PBS-injected control mice in

both strains. We concluded that FcγRIIB did not influence the antitumor effect mediated by the Chi-Tn mAb in this tumor model.

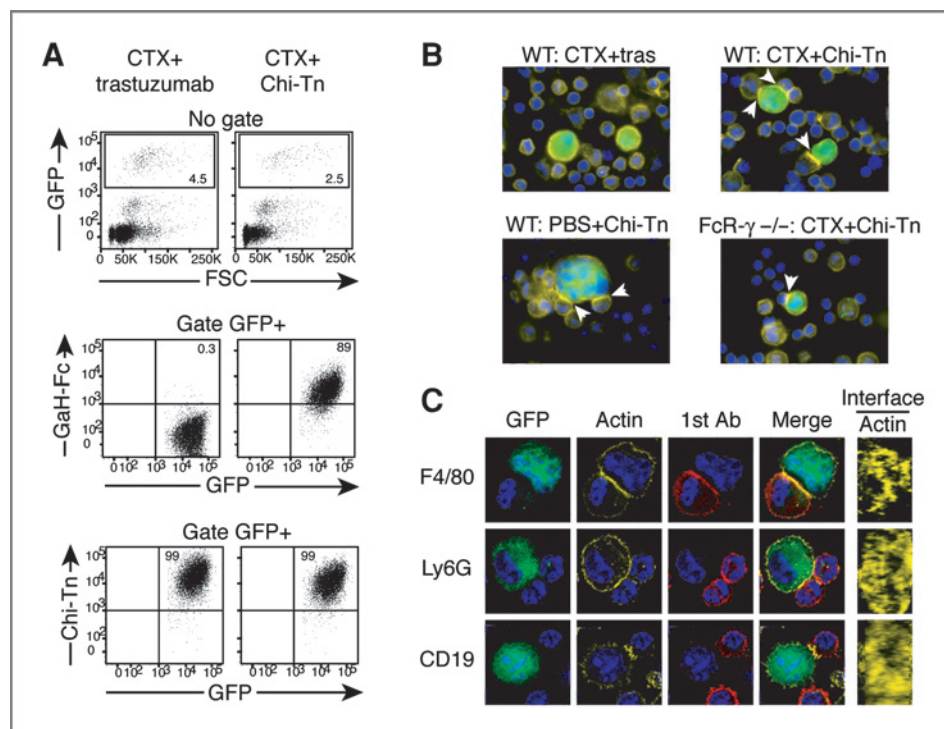
The activating FcγR, responsible for ADCC *in vitro*, were shown to contribute substantially to antibody-mediated tumor growth inhibition *in vivo*, since the antitumor effect of rituximab and trastuzumab were impaired in mice deficient for the FcR-γ chain (FcR-γ<sup>-/-</sup> mice), which lack functional FcγRI, FcγRIII, and FcγRIV (4, 5). The survival of FcR-γ-deficient mice grafted with TA3Ha cells and treated with CTX + Chi-Tn was dramatically decreased compared with wild-type mice (20% and 80% survival respectively,  $P = 0.02$ , compare Fig. 4B, left and right panels), and did not differed from control FcR-γ<sup>-/-</sup> mice injected with CTX + PBS (Fig. 4B, right panel, 20% and 0% survival respectively,  $P = 0.373$ ). These results show that activating FcγRs play a critical role in the therapeutic antitumor effect of CTX + Chi-Tn mAb, supporting a role for ADCC in tumor rejection induced by Chi-Tn.

#### TA3Ha cells form conjugates *in vivo* with effectors of the tumor microenvironment after CTX + Chi-Tn mAb treatment

To further investigate the mechanisms of Chi-Tn mAb-induced tumor rejection *in vivo*, BALB/c mice were given TA3Ha-GFP<sup>+</sup> cells on day 0, CTX on day 1, and then Chi-Tn mAb or trastuzumab on day 2. Four hours after mAb injection, peritoneal cells were harvested and analyzed by flow cytometry. As shown in Figure 5A, Tn-positive TA3Ha-GFP<sup>+</sup> cells from Chi-Tn mAb but not from trastuzumab-treated mice were labelled with the secondary GaH-Fc-PE antibody, showing that the Chi-Tn mAb was bound to the plasma membrane of TA3Ha cells *in vivo*. Analysis of these peritoneal infiltrates by deconvolution microscopy showed conjugates between GFP<sup>+</sup> tumor cells and cells of the tumor microenvironment in CTX + Chi-Tn-treated mice, but not in control mice



**Figure 4.** Chi-Tn mAb induces TA3Ha ADCC *in vitro* and FcγR-dependant growth inhibition *in vivo*. A, IFNγ-activated RAW effector cells plated at different E/T ratios were incubated for 24 hours with Chi-Tn mAb (squares) or trastuzumab (circles) at 20 μg/mL or medium alone, together with CFSE-labelled TA3Ha or SKBR3 targets. Percentages of specific lysis are reported for each ratio. B, mice were given TA3Ha cells (day 0), treated with CTX (50 mg/kg, day 1) when indicated, then treated from day 2 with the Chi-Tn mAb at 20 mg/kg or with PBS (6 total injections). Filled triangles, CTX + Chi-Tn; open triangles, Chi-Tn; filled circles, CTX + PBS. Left: wild-type BALB/c mice ( $n = 5$ /group). Middle: FcγRIIB<sup>-/-</sup> mice ( $n = 10$ /group). Right: FcR-γ<sup>-/-</sup> mice ( $n = 15$ /group).



**Figure 5.** Conjugates between TA3Ha and immune cells after CTX + Chi-Tn treatment *in vivo*. BALB/c mice were grafted i.p. with TA3Ha-GFP cells ( $10^6$ /mouse) on day 0. PBS or CTX (50 mg/kg) was given on day 1, and Chi-Tn mAb ( $n = 3$ ) or trastuzumab ( $n = 3$ ) were injected at 20 mg/kg on day 2. Peritoneal cells were harvested 4 hours after mAbs injection. **A**, peritoneal cells were labelled with GaH-Fc-PE or with Chi-Tn + GaH-Fc-PE. GFP fluorescence was examined by flow cytometry in ungated cells (top). GaH-Fc-PE labelling (middle) and Chi-Tn + GaH-Fc-PE labelling (bottom) were examined in the GFP<sup>+</sup> gate. Numbers represent percentages of cells in the corresponding gate or quadrant. **B**, peritoneal cells from WT mice or from FcR- $\gamma^{-/-}$  mice treated with the indicated combination were labelled with phalloidin (yellow) and DAPI (blue). Green, TA3Ha-GFP cells. **C**, peritoneal cells were labelled using the indicated primary antibody (1st Ab, red) and phalloidin (yellow). Green, TA3Ha-GFP cells; blue, DAPI. Merge images represent close contacts between TA3Ha-GFP<sup>+</sup> and immune cells. 3D analysis of the interface was done, and the z, x plane represented. One representative analysis of several conjugates is shown.

injected with trastuzumab (Fig. 5B top panels and Fig. 5C). F-Actin accumulated at the interface between host peritoneal cells and tumor cells suggesting synapse formation *in vivo*. Macrophages (F4/80<sup>+</sup>) and neutrophil/polymorphonuclear cells (Ly6G<sup>+</sup>), as well as B cells (CD19<sup>+</sup>), were found in conjugation with tumor cells, at a mean frequency around 30% for each cell type (Fig. 5C). No interactions with tumor cells were observed for NK cells (DX5<sup>+</sup>), T cells (CD3<sup>+</sup>), dendritic cells (CD11c<sup>+</sup>), nor mastocytes or basophils (FcεRI<sup>+</sup>; data not shown).

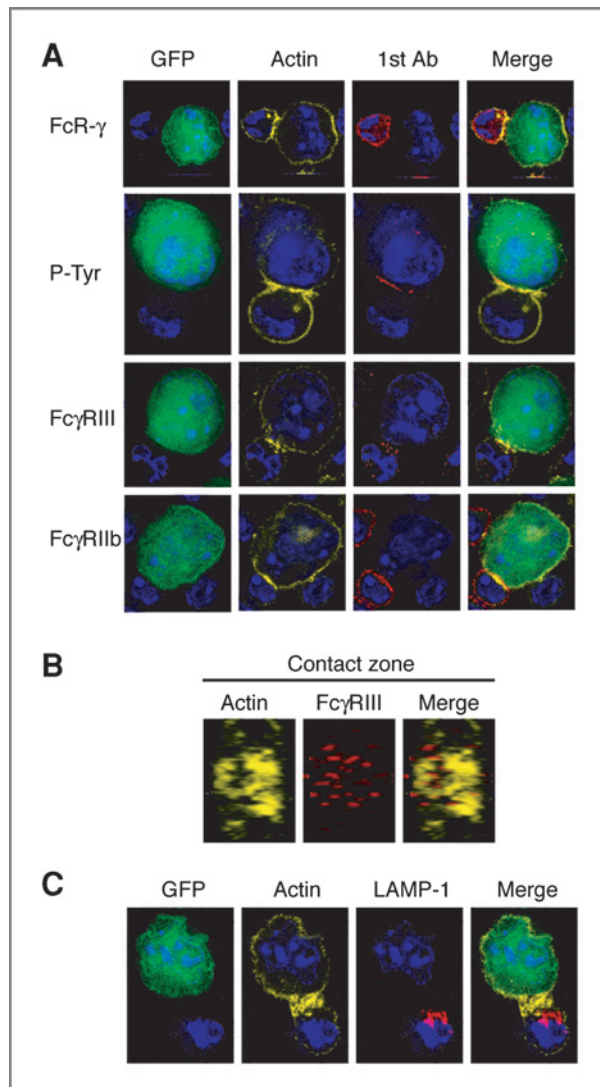
Orthogonal projections of the contact zone between potential effectors and GFP<sup>+</sup> tumor cells showed that F-actin was reorganized into dense-punctuated structures across the interfaces, with one or several small zones with no F-actin (see the representative "en face" views in Fig. 5C), forming what has been referred to as "actin rings," similarly to immunologic T-cell synapses (30). In addition, FcγRIII, one of the most likely effectors of ADCC, accumulated at the interface within the actin "wholes" (Fig. 6A and B). FcR-γ chain and FcγRIIB were also present at the interface but did not accumulate in this zone (Fig. 6A). The marked accumulation of tyrosine phosphorylated proteins at the interface suggested that active signaling was occurring between the 2 cells. We also observed the polarization of LAMP-1<sup>+</sup> vesicles near the

interface, again suggesting that these structures represent functional cytotoxic synapses (Fig. 6C). Thus, organized macromolecular structures formed at the interface between ADCC effector cells and tumors cells upon injection of the Chi-Tn mAb. These structures are organized in central and peripheral supra-molecular activation clusters (SMAC) that, by analogy to the structures formed at the interface between T cells and antigen-presenting cells, could be called "ADCC synapses."

Interestingly, conjugates between tumor cells and macrophages, neutrophils, or B cells could still be observed in FcR-γ-deficient mice (lacking activating FcγR) after CTX + Chi-Tn mAb treatment, probably because of the engagement of the inhibitory FcγRIIB present on these effector cells. Synapses between tumor and effector cells were also present in wild-type mice treated with Chi-Tn mAb in the absence of CTX (Fig. 5B lower panels, and Supplementary Fig. S6). Under these 2 last experimental conditions, no antitumor effect was observed (see Fig. 3A and Fig. 4B), indicating that the cross-linking of the Chi-Tn mAb is not by itself sufficient to induce tumor cell death *in vivo* (activation of the effector cells is required).

We conclude that macrophages and neutrophils are the 2 main cytotoxic cell types that mediated Chi-Tn-induced





**Figure 6.** Analysis of the ADCC synapse between TA3Ha and immune cells. Mice were grafted with TA3Ha-GFP<sup>+</sup> cells, injected with CTX and treated with Chi-Tn mAb or trastuzumab as in Figure 5. Peritoneal cells were labelled 4 hours after mAb injection using the indicated 1st Ab (red) against: FcR- $\gamma$  chain, phosphotyrosine (P-Tyr), Fc $\gamma$ RIII or Fc $\gamma$ RIIb (A) or LAMP-1 (C), and phalloidin (yellow). Green, TA3Ha-GFP cells; blue, DAPI. One representative analysis of several conjugates is shown for each marker. B, synapses between Fc $\gamma$ RIII<sup>+</sup> cells and TA3Ha-GFP<sup>+</sup> cells were reconstructed in the z, x plane. Yellow, phalloidin; red, Fc $\gamma$ RIII. One representative experiment is shown.

TA3Ha rejection by ADCC, and that ADCC *in vivo* occurs through organized ADCC synapses that form at the interface between effectors and tumor cells.

## Discussion

The relative contribution of direct growth inhibition, indirect cytotoxicity (ADCC or CDC) and adaptive immunity (2, 6, 31) in the therapeutic effect of mAbs is still a matter of sustained investigation. We show here that a mAb (Chi-Tn)

induced tumor rejection *in vivo* through ADCC, in the absence of any detectable direct effect on tumor growth *in vitro*.

The Chi-Tn mAb is specific for the GalNAc-O-Ser or Thr epitope expressed on various proteins of the mucin family in both humans and mice carcinomas (12, 27). Distinct anti-Tn mAbs display differences in their fine specificity (the binding is affected by the density of the Tn determinant or/and by the amino acid residues neighboring O-glycosylation sites) and show different cancer recognition patterns and variable effects on tumor growth (16, 32–36). With the exception of Jurkat T cells, the Chi-Tn mAb did not affect the growth of any of the Tn-expressing tumor cells tested, particularly the breast tumor cell line TA3Ha that was used here (Fig. 2, and Supplementary Fig. S4).

Chi-Tn-induced tumor rejection *in vivo* required pretreatment of mice with CTX. TA3Ha cells were insensitive to PhM (the active metabolite of CTX) *in vitro* (28, 29, Fig. 3D), and *in vivo* (Fig. 3A). It is unclear thus far by which mechanism CTX participated to the antitumor effect of the Chi-Tn mAb *in vivo*. PhM did not potentiate Chi-Tn to induce TA3Ha cell death or ADCC *in vitro* (Fig. 3D and Supplementary Fig. S5). The other hypothesis is that CTX modified the host microenvironment favoring the antitumor effect of Chi-Tn. Indeed, CTX induces the release of cytokines from bone marrow cells (37, 38), which could activate ADCC effectors, thereby promoting tumor rejection. This idea is strengthened by the results in Figure 5B and Supplementary Figure S6, showing that synapses are not sufficient by themselves in the absence of CTX to induce tumor cell death.

Tumor growth inhibition by the CTX + Chi-Tn mAb combination *in vivo* needed activating Fc $\gamma$ R (Fig. 4B). Similarly, to the FcR- $\gamma$  chain-dependent antitumor effect of rituximab and trastuzumab (4–6), Chi-Tn mAb cross-linking was not sufficient to kill TA3Ha cells *in vitro* and *in vivo* (Figs. 2A, 3A, and 5B and Supplementary Fig. S6). Instead, our results suggest that TA3Ha cells are eliminated upon CTX + Chi-Tn mAb treatment through FcR- $\gamma$  chain-dependant ADCC (Figs. 4–6). Moreover, no enhanced response to Chi-Tn mAb alone was observed in Fc $\gamma$ RIIb-deficient mice, as compared with wild-type (WT) mice (Fig. 4B), contrary to that was described for rituximab and trastuzumab (4). This result suggests that the CTX pretreatment used here may overcome the negative effect of Fc $\gamma$ RIIb *in vivo*.

No direct demonstration that ADCC occurs *in vivo* was reported so far. Indirect evidence showing an infiltration of the tumors by Fc $\gamma$ R<sup>+</sup> lymphoid cells in patients treated with trastuzumab was only reported (11). In this study, we show for the first time that hematopoietic cells were conjugated to tumor cells *in vivo* in Chi-Tn but not in control trastuzumab-treated mice. 3D deconvolution immunofluorescence microscopy showed that the interface between the 2 cells was organized into SMACs, defining what could be referred to as "ADCC synapses" *in vivo*. This study represents one of the few direct demonstrations of the existence of immune synapses *in vivo*. The analysis of these conjugates also identified the nature of the effector cells involved in tumor rejection through ADCC *in vivo*. Surprisingly, no NK cells were found in the conjugates, while F4/80<sup>+</sup> macrophages, Ly6G<sup>+</sup> neutrophils and CD19<sup>+</sup> B cells were present in the same proportion in the doublets. Macrophages



and neutrophils were already reported to mediate tumor cell killing through ADCC and synapse formation between these effectors and tumor cells was shown *in vitro* (39, 40). Moreover, B cells were also suggested to be necessary for tumor growth inhibition *in vivo* (41).

Chemotherapies synergize with therapeutic mAbs (24, 25) by interfering with DNA-repair processes (42), by increasing CTL-mediated tumor cell lysis (43), or decreasing regulatory T cells (44). Moreover, some reports (26, 31) showed that the timings of chemotherapy and mAb injections are important for the duration of the antitumor response. Our model using CTX before Chi-Tn mAb treatment reinforce this idea, suggesting that a tumor-specific memory response may also be involved in the CTX + Chi-Tn mAb antitumor effect.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Antibody-Dependent Cell Cytotoxicity Synapses Form in Mice during Tumor-Specific Antibody Immunotherapy

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