Supplementary Methods

Generation of TA3Ha-GFP cells

TA3Ha-GFP cells were generated by infection of TA3Ha cells with 10 MOI of the pWPXLd lentivirus coding for the eGFP sequence (from D. Trono, EPFL, Lausanne, Switzerland). Cells were sorted on a FACSVantage cell sorter (BD Biosciences, San Jose, CA), to obtain a homogeneous population containing more than 95% of Tn+ GFP+ cells.

Production of the Chi-Tn mAb

The variable V fragments of the murine parental anti-Tn mAb 83D4 (IgM) heavy and light chains were inserted into expression vectors containing the constant regions of the human Cγ1 heavy chain and of the human κ light chain respectively. The chimera was expressed by transfection of the plasmids into the X63 myeloma cell line (20). As indicated in (20), the affinity of the IgG1 anti-Tn chimera measured by Surface Plasmon Resonance spectroscopy (Biacore) against a glycopeptide containing two consecutive Tn antigens was lower (2.2 x 10^{-8} M) compared to the affinity of the native mouse IgM anti-Tn mAb 83D4 (3.1 x 10^{-9} M). Similar results were obtained when using a glycopeptide containing three consecutive Tn-antigens: the measured affinity constant was 5.1 x 10^{-9} for the parental mouse 83D4 and 2.1 x 10^{-8} for the chimeric IgG1 anti-Tn mAb. We selected a high-producer sub-clone by limiting dilutions, which was then grown in RPMI 1640 medium containing 10% of immunoglobulins-depleted FCS and neomycin. The Chi-Tn mAb was purified from the culture supernatants on a protein A column, then desalted on PD-10 columns (GE Healthcare, Orsay, France) and recovered in PBS. Endotoxins were finally removed using a Mustang® E membrane (Pall Corporation, Port Washington, NY).

Three-dimensional Deconvolution microscopy

Peritoneal cells were recovered by washing using 5 ml of RPMI containing 10% FCS and heparin, and were carefully resuspended in RPMI-3% FCS. Cells (10^6/sample) were allowed to adhere on to poly(L-lysine)-coated glass coverslips for 30 min at 37°C. Cells were then fixed with 4% paraformaldehyde for 10 min at room temperature, quenched with 100 mM glycine in PBS, and permeabilized with PBS containing 0.05% saponin and 0.2% BSA. Cells were incubated with the indicated primary antibody and/or with phalloidin coupled to Alexa Fluor 546 (Molecular Probes) for 30 min, washed and labelled with the corresponding fluorochrome-conjugated secondary antibody. Biotinylated F4/80 was purchased from Biolegend (San Diego, CA). Anti-Ly6G, anti-CD19-APC and anti-LAMP-1 were obtained from BD Biosciences, rabbit Anti-FcR-γ and anti-phosphotyrosine 4G10 from Upstate Biotechnology (Lake Placid, NY), anti-mouse FcγRIII from R&D Systems (Minneapolis, MN). The anti-FcγRIIb Ly17.2 was kindly given by U. Hammerling (MSKCC, NY). Fab'2 donkey anti-Rat-Cy5 and donkey anti-rabbit-Cy5 were obtained from Jackson. Phalloidin-Alexa Fluor 546 and streptavidin-Alexa Fluor 647 were from Molecular Probes. Cells were then stained with DAPI, and mounted on to glass slides with Mowiol. Images were acquired using an Eclipse 90i upright microscope (Nikon Instruments Europe, Amstelveen, Netherlands) equipped with a CDD camera (CoolSNAP HQ2, Roper Scientific, Trenton, NJ), a Piezo flexure objective scanner (Physik Instrumente, Karlsruhe, Germany), and an oil-immersion objective (x100 CFI Plan Apo VC, NA 1.4). Deconvolutions were performed on stacks of images taken with a 0.2 μm plane spacing, using the three-dimensional deconvolution module of MetaMorph software (Universal Imaging Corp., Downingtown, PA) and the fast iterative constrained PSF-based algorithm. For three-dimensional analysis, images acquired in the z-axis were integrated and rotated using MetaMorph, and synapses were reconstructed in the z, x plane.

Supplementary Figure Legends

Supplementary Fig. S1 : Quantification of ADCC *in vitro* by flow cytometry.

RAW cells primed with 12 ng/ml of recombinant mouse IFNγ (R&D systems, Minneapolis, MN) overnight at 37°C were used as effector cells and plated at ratio 25/1 in a round bottom 96-W plate in the presence of Chi-Tn mAb or trastuzumab (20 µg/ml final concentration). Target TA3Ha cells labeled with 5 µM CFSE (Molecular Probes) were distributed at 10^4 cells/well. Co-cultures were incubated at 37°C for 24h, and then analyzed by flow cytometry. Cells were harvested and labeled with DAPI. Flow-Count™ Fluorospheres (10^4/sample, Coulter Corporation, Miami, FL) were added just before each sample acquisition and mixed. After gating, $5x10^3$ beads were acquired. The absolute number of CFSE-positive TA3Ha surviving cells was determined for each sample in the DAPI-negative living cells gate, and the mean of the triplicate was calculated. The % of specific lysis was calculated as follow: (1- Absolute number of surviving experimental target cells/ Absolute number of surviving control target cells) x100. A representative experiment is shown. Absolute numbers of surviving CFSE+ TA3Ha cells are indicated. Percentages of specific lysis are 6,6% and 48,8% for trastuzumab and Chi-Tn mAb respectively.

Supplementary Table 1 : Tn expression at the plasma membrane of patients' ascitis ovarian tumour cells

* Ascitis samples from ovarian cancer patients were labelled with anti-Epcam-FITC, anti-CD45-PerCP-Cy5.5 and biotinylated-Chi-Tn mAb or biotinylated-Chi-Tn mAb pre-incubated with GalNAc as described in Materials and Methods, or with biotinylated-human Immunoglobulins (TEGELINE®, LFB, Courtaboeuf, France) as an isotype control. After labelling with streptavidine-PE, living cells were acquired by FACS. Epcam+CD45- tumour cells were gated, and the PE Mean Fluorescence Intensity (M.F.I.) was determined.

§ Given the variability of the background MFI for the isotype control, samples were considered as positive for Tn expression when the ratio MFI Tn /MFI Tn+GalNAc was > 3. Among 23 patients studied, 17 (74%) were clearly Tn-positive.

(+): Tn positive; (-):Tn negative; (+/-): Tn slighlty positive.

Supplementary Fig. S2 : Chi-Tn mAb binding to Jurkat and TA3Ha cells.

Jurkat or TA3Ha cells were labelled in FACS buffer with the Chi-Tn mAb at the indicated concentrations for 15 min on ice, washed, then labelled using a secondary F(ab')2 goat anti-human IgG Fc anti-serum coupled to phycoerythrin (GaH-Fc-PE). After washing, cells were analyzed by flow cytometry, and the PE mean fluorescence intensity (M.F.I.) was determined. For both Jurkat and TA3Ha cells, Chi-Tn mAb binding increases rapidly from 0 to 20 µg/ml, then reach a plateau from 70 µg/ml.

Supplementary Fig. S3 : Jurkat cell death after Chi-Tn mAb cross-linking.

We determined whether Jurkat cell death was due to a hyper cross-linking of the Tn antigen by Chi-Tn mAb + goat anti-human IgG Fc anti-serum (GaH), or was due to an increased avidity of the Chi-Tn mAb when it was bound to the GaH. For this purpose, Jurkat cells were seeded at 100,000 cells/well in a 96-well plate. The Tn antigen was then cross-linked by three different ways:

In the *upper panel* ("Hyper cross-linking"), Jurkat cells were incubated for 15 min on ice with the Chi-Tn mAb (empty squares) or with rituximab (empty circles) as an isotype control at the indicated final concentration, washed, then the GaH was added at 100 µg/ml final concentration.

In the *middle panel* ("Chi-Tn immune complexes"), the Chi-Tn mAb (empty squares) or the rituximab (empty circles) at the indicated final concentration were pre-incubated for 30 min at

37°C with the GaH at 100 µg/ml (final concentration) to allow immune complexes to form. Immune complexes were then added to Jurkat cells.

Finally, in the *lower panel* ("Cross-linking") Jurkat cells were treated as in Figure 2A: cells were incubated for 15 min on ice with the Chi-Tn mAb (empty squares) or with rituximab (empty circles) as an isotype control at the indicated final concentration, then the GaH was added without any washing at 100 µg/ml final concentration. Cells were in parallel incubated with Chi-Tn mAb alone (filled squares) or rituximab alone (filled circles).

All the different plates were incubated for 24 h at 37°C. Cells were then recovered, labelled with propidium iodide (PI), and the percentage of PI+ dead cells was determined for each sample by FACS.

The results showed similar proportions of Jurkat cell death when the Tn antigen was cross-linked using the Chi-Tn mAb then GaH without washing (panel "Cross-linking"), or using pre-formed Chi-Tn immune complexes (panel "Chi-Tn immune complexes"). However, the percentages of Jurkat cell death were lower when the cells were hyper cross-linked by the GaH after washing Chi-Tn mAb-labelled cells (panel "Hyper cross-linking"). These results thus suggest that the anti-human antibody GaH increased the binding avidity of the Chi-Tn mAb, and consequently, cell death.

Supplementary Fig. S4 : Proliferation of MCF-7 and SHIN-3 cultured with cross-linked Chi-Tn mAb.

The MCF-7 cell line (human breast cancer) was provided by M.F. Poupon, Institut Curie, France. SHIN-3 cells (human ovarian cancer) were a generous gift from C. Cabella (Bioindustry Park del Canavese, Ivrea, Italy) who obtained them from Y. Kiyozuka (Kansai Medical University, Osaka, Japan).

A) Tn antigen expression at the plasma membrane of MCF-7 and SHIN-3 cells. Cells were labelled in FACS buffer using the Chi-Tn mAb or human immunoglobulins (Ig) as isotype control at 20 µg/ml, washed, then revealed with the GaH-Fc-PE. Numbers indicate the percentage of cells in the quadrant.

B) Proliferation of MCF-7 and SHIN-3 cells in the presence of the Chi-Tn mAb. MCF-7 (white bars) or SHIN-3 cells (black bars) were cultured at 10,000 cells/well for 72 h in a 96-well plate with the Chi-Tn mAb (20 µg/ml) in the presence or absence of the secondary goat anti-human IgG Fc anti-serum (GaH). Cell cultures with doxorubicine at 1 µg/ml were used as positive control of cell death. Cell proliferation was expressed as a percentage of inhibition compared to untreated cells cultured in medium alone. No significant inhibition of cell proliferation was observed after culture of the Tn-positive cell lines MCF-7 and SHIN-3 with Chi-Tn mAb alone or with cross-linked Chi-Tn mAb.

Supplementary Fig. S5 : CTX/PhM does not sensitize TA3Ha cell death by Chi-Tn mAb-mediated ADCC *in vitro*.

TA3Ha cells were cultured at 37°c for 24 h in the presence of CTX or its active metabolite phosphoramid mustard (PhM) at 30 µM (the peak of PhM obtained *in vivo* after injection of 50 mg/kg of CTX i.p.). Cells were then recovered, labelled with CFSE, and were incubated for 24 h at 37°C at the indicated E/T Ratio with the RAW effector cells in the presence of the Chi-Tn mAb (20 µg/ml, white bars) or of trastuzumab as an isotype control (black bars). The number of TA3Ha-CFSE cells present in each well was then determined by FACS as described in the Materials and Methods section.

Supplementary Fig. S6 : Presence of synapses *in vivo* in experimental conditions where Chi-Tn mAb does not reject TA3Ha tumour cells.

Synapses are present in vivo even when Chi-Tn mAb does not reject tumour cells.

A) Wild-type (WT) BALB/c mice were grafted with TA3Ha cells (day 0), injected with PBS (day 1), then treated with Chi-Tn mAb or trastuzumab (20 mg/kg, day 2). Four hours after mAb injection, peritoneal cells were plated on glass coverslips and labelled as followed: *Upper panel*: cells were labelled with phalloidin-Alexa Fluor 546 (yellow), stained with DAPI (blue). Green, TA3Ha-GFP cells. *Lower panel*: cells were labelled using the indicated primary antibody (1st Ab). Cells were then labelled with phalloidin-Alexa Fluor 546 (yellow), and with the corresponding secondary antibody coupled to Cy5 or with streptavidin-Alexa Fluor 647 (red). Green, TA3Ha-GFP cells; blue, DAPI. Cells were analyzed by deconvolution microscopy. Merge images show contacts between TA3Ha-GFP+ target cells and immune cells. One representative analysis of at least five conjugates is shown.

B) FcR-γ chain deficient mice were grafted with TA3Ha cells (day 0), injected with CTX (50 mg/kg, day 1), then treated with Chi-Tn mAb or trastuzumab (20 mg/kg, day 2). Four hours after mAb injection, peritoneal cells were harvested and treated as above.