Supplementary data

The fully synthetic glycopeptide MAG-Tn3 therapeutic vaccine induces tumor-specific cytotoxic antibodies in breast cancer patients

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The PDF file includes:

Supplementary Materials and Methods

Determination of total IgG1, IgG2, IgG3, and IgG4 concentration

The concentration of IgG1, IgG2, IgG3, and IgG4 was determined by ELISA using the human IgG subclass profile kit (Fischer Scientific). Sera from patients were tested at a 1:2500 dilution. The optical densities were recorded at 450 nm using a Multiskan spectrum (Fischer Scientific) plate reader and the concentrations calculated with 4PL regression from standard calibration curves of control samples provided with the kit.

Antibody-Dependent Cytotoxicity Assay

After centrifugation using histopaque-1077 (Sigma Aldrich) to remove debris, Jurkat, MCF7, SKBR3, or MDA231 cells were labeled with 25 µM CFSE (carboxyfluorescein succinimidyl ester, Life Technologies) for 8 min. Cells were then incubated for 4 h with 250 µg/mL purified immunoglobulins from patients and $CD56^+$ primary NK cells (ATCC) at different effector: target ratios (3:1, 1:1, 0.3:1, no NKs). After incubation, cells were stained with Live/Dead blue fixable stain (Fischer Scientific) for 15 min at 4°C. Cells were then fixed using 100 µL BD Cytofix (BD) and data acquired using an LSR Fortessa cytometer. Data were analyzed using FlowJo software.

Hematology and PBMC phenotyping of immune cells

Complete blood counts were determined using fresh whole blood, whereas immune cell phenotyping was performed with frozen vials of 5.10_6 PBMCs. PBMCs were thawed in a 37°C water bath and washed in 40 mL warm RPMI 1640 medium supplemented with 10 % FCS and antibiotics. Cells were then stained with Live/Dead blue fixable stain® for 15 min at 4°C. After washing, cells were stained with the following mouse anti-human antibodies: CD3-PercpCy5.5 (Clone SK7, BD), CD4-V500 (Clone RPA-T4, BD), CD8-PE-Cy7 (Clone RPA-T4, BD), CD20-BUV737 (Clone RPA-T4, BD), CD56-APC (Clone NCAM16.2, BD), CD19-BV786 (Clone SJ25C1, BD), IgD-BB515 (Clone IA6-2, BD), CD38-BV421 (Clone HIT2, BD), CD27-AF700 (Clone M-T271, BD), CD14-APCH7 (Clone MΦP9, BD), CD16-PECF594 (Clone 3G8, BD), HLA-DR-BUV395 (Clone G46-6, BD), CD11c-PE (Clone SHCL3, BD), and CD123-BV605 (Clone 9F5, BD) in brilliant stain buffer (BD). Cells were fixed using 100 µL BD cytofix (BD) and data acquired using an LSR Fortessa cytometer. Data were analyzed using FlowJo software.

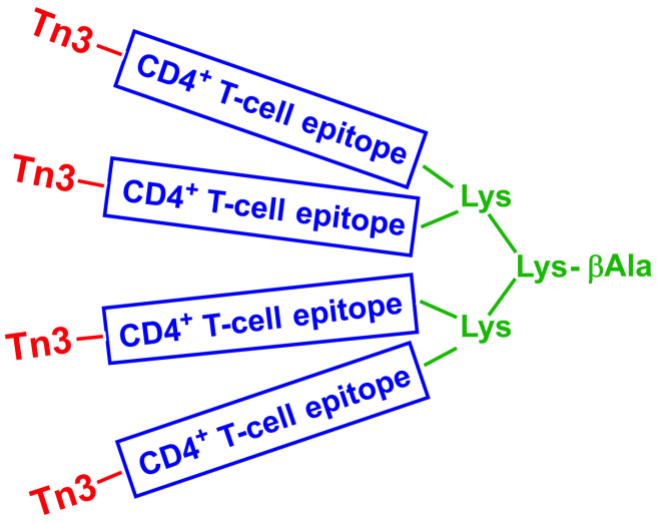


Figure S1. Schematic representation of the MAG-Tn3 vaccine. Tn3: H-Ser(α-D-GalNAc)-Thr(α–D-GalNAc)-Thr(α-D-GalNAc), carbohydrate tumor-associated antigens (red). $CD4_+$ T cell epitope: tetanus toxin peptide $TT_{830-844}$ (blue).

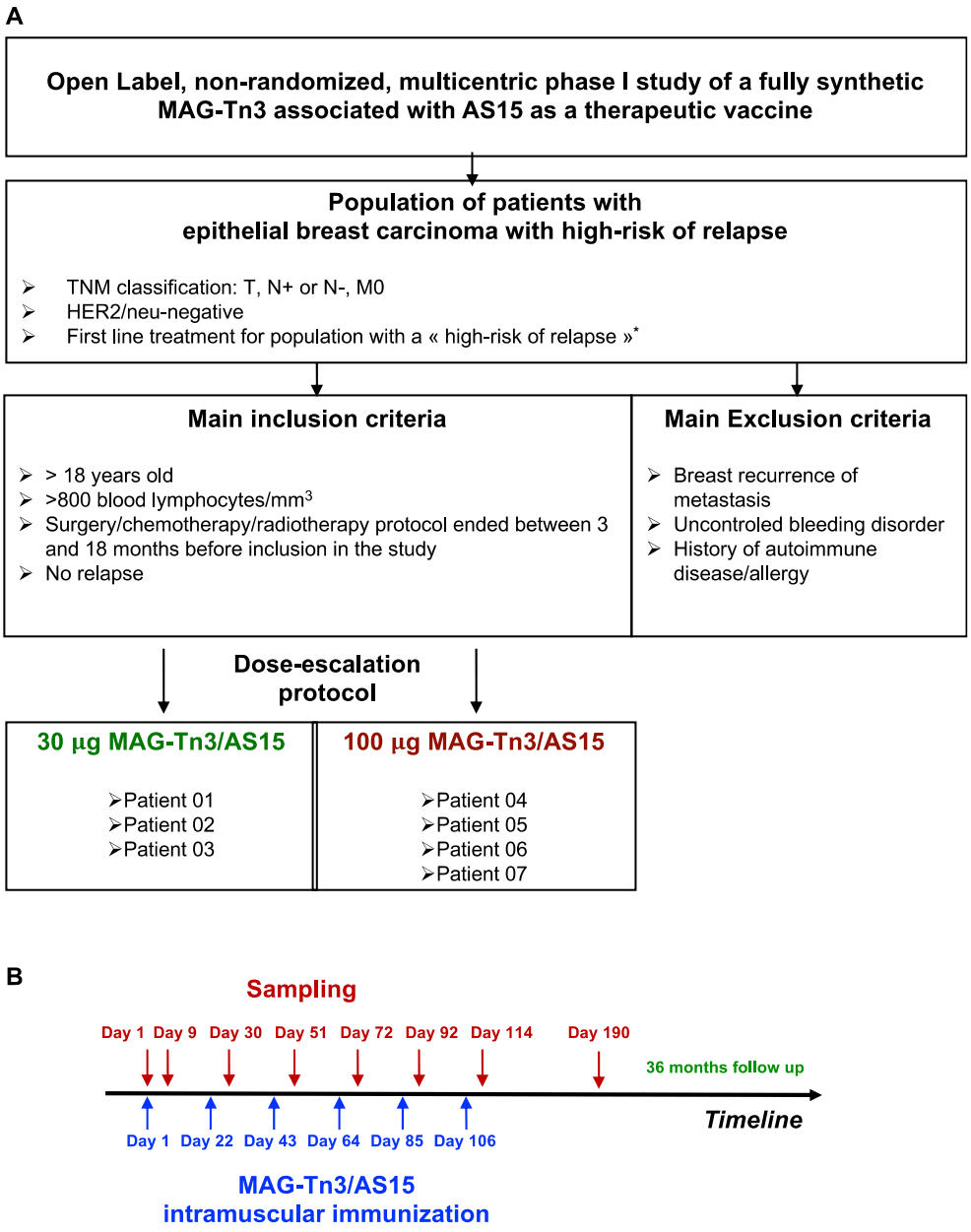


Figure S2. Design of the phase I clinical trial MAGTRIVACSEIN. (**A**) Clinical trial organization and the main inclusion and exclusion criteria. (**B**) Study organization schedule showing the MAG-Tn3/AS15 administration (bottom) and blood sampling scheme (top).
*Patients were defined as being at high risk of relapse if they were either positive for hormone receptors (ER+ and/or PR+ ≥1 0%, defined by immunohistochemistry), with at least one positive lymph node at primary surgery or after completion of 6 to 8 cycles of anthracycline/taxane-based neoadjuvant chemotherapy, or negative for hormone receptors (< 10%, defined by immunohistochemistry), *i.e.* « Triple Negative Cancer ».

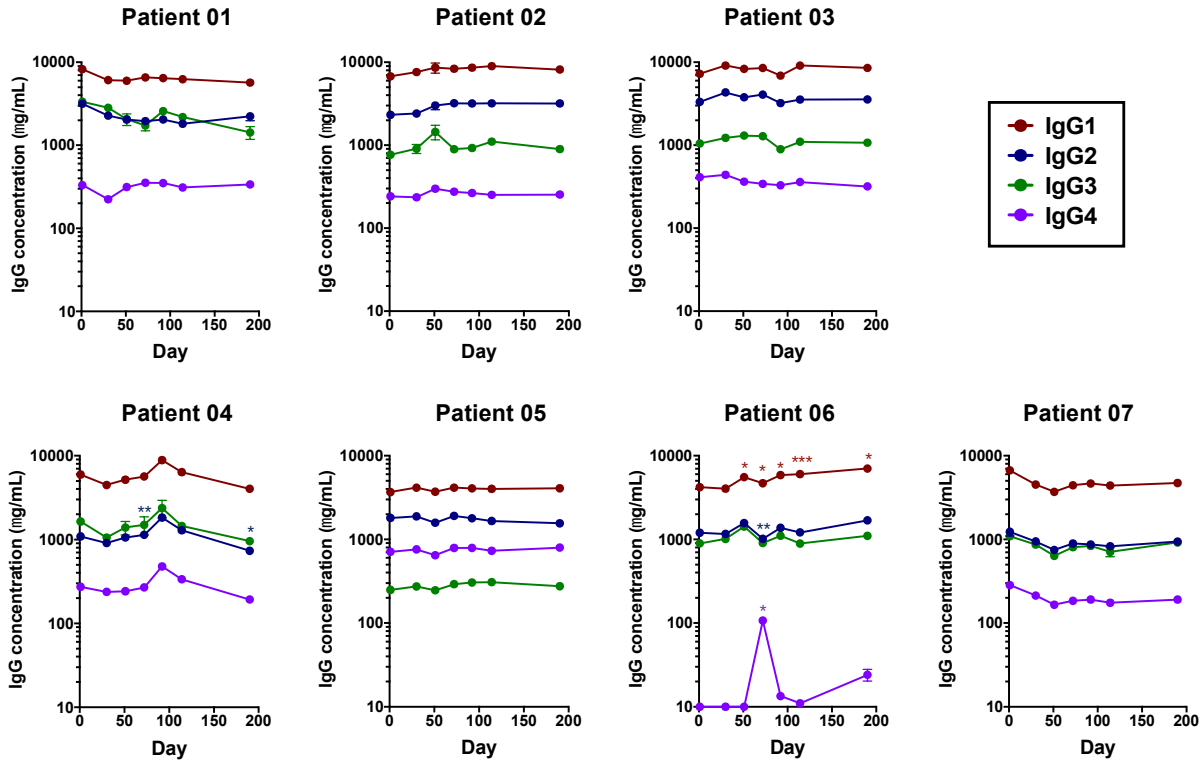
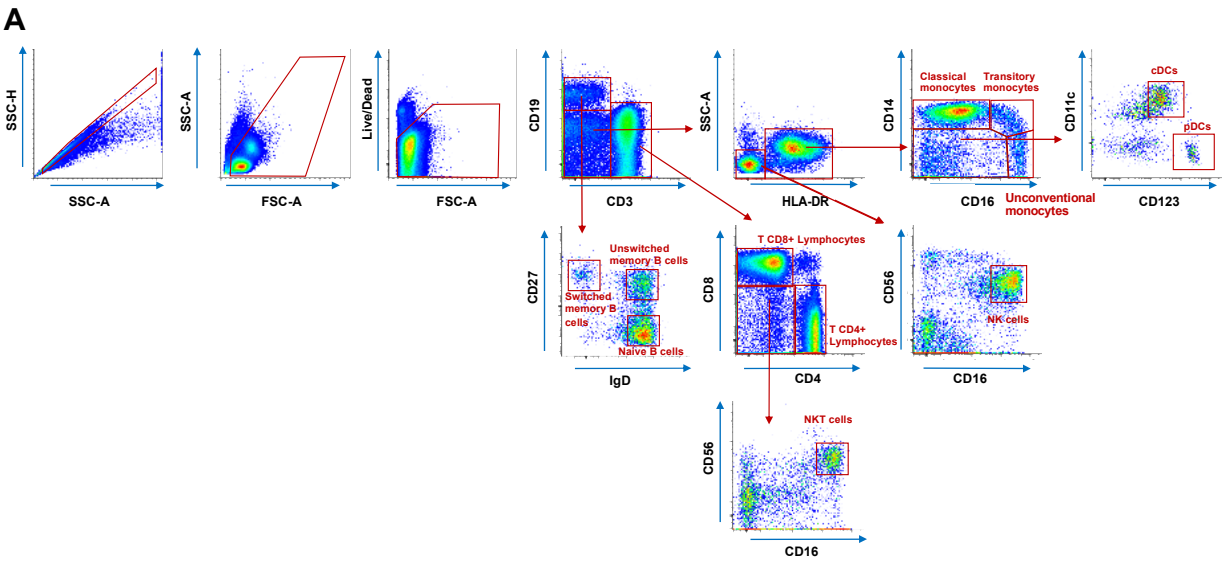
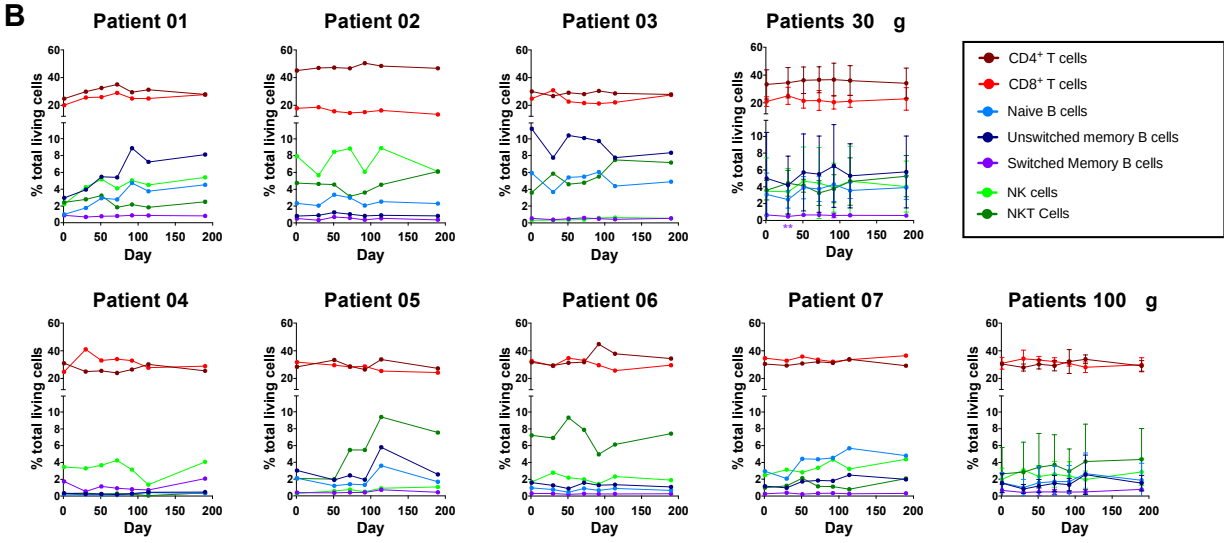


Figure S3. Immunization of breast cancer patients with the MAG-Tn3 vaccine formulated with AS15 does not alter the concentration of seric IgG1, IgG2, IgG3 and IgG4. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. Sera were collected at various times after immunization and tested for total IgG1, IgG2, IgG3, and IgG4 concentration by ELISA. Data are expressed as the mean ± SD of quadruplicates from two independent experiments. The statistical significance of the differences was determined by the unpaired Student's t-test relative to baseline. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.


A
SSC-H
SSC-A
SSC-A
FSC-A
Live/Dead
FSC-A
CD19
CD3
SSC-A
HLA-DR
CD14
Classical monocytes
Transitory monocytes
CD16
Unconventional monocytes
CD11c
cDCs
pDCs
CD123
CD27
Unswitched memory B cells
Switched memory B cells
Naive B cells
IgD
CD8
T CD8+ Lymphocytes
T CD4+ Lymphocytes
CD4
CD56
NK cells
CD16
CD56
NKT cells
CD16


B
Patient 01
Patient 02
Patient 03
Patients 30 g
Patient 04
Patient 05
Patient 06
Patient 07
Patients 100 g
% total living cells
Day
CD4+ T cells
CD8+ T cells
Naive B cells
Unswitched memory B cells
Switched Memory B cells
NK cells
NKT Cells

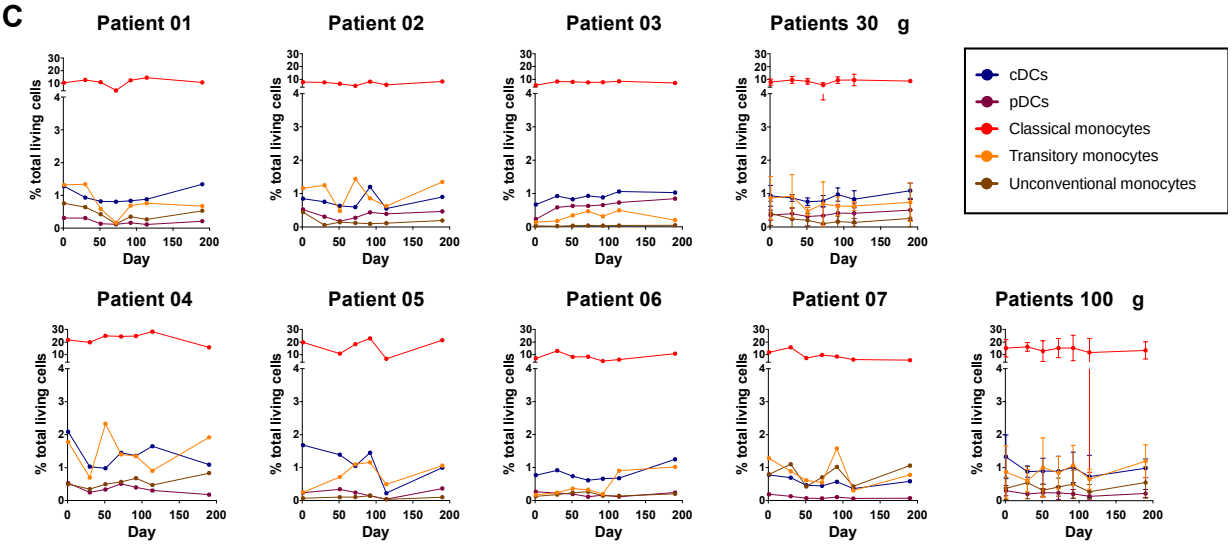

C
Patient 01
Patient 02
Patient 03
Patients 30 g
Patient 04
Patient 05
Patient 06
Patient 07
Patients 100 g
% total living cells
Day
cDCs
pDCs
Classical monocytes
Transitory monocytes
Unconventional monocytes

Figure S4. Analysis of immune cell populations in PBMCs of patients immunized with the MAG-Tn3 vaccine. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. The percentage of blood myeloid and lymphoid subsets was determined on frozen PBMCs by flow cytometry. Data are expressed as (the number of the gated cells divided by the total number of living cells) x 100. (**A**) Gating used to characterize the immune cell populations was performed as follows: naïve B cells: $CD3^-$, $CD19^+$, IgD^+, $CD27^-$; unswitched memory B cells: $CD3^-$, $CD19^-$, IgD^+, $CD27^+$; switched memory B cells: $CD3^-$, $CD19^-$, IgD^+, $CD27^+$; $CD8^+$ T cells: $CD3^+$, $CD19^-$, IgD^+, $CD4^-$, $CD8^+$; $CD4^+$ T cells: $CD3^+$, $CD19^-$, IgD^+, $CD4^+$, $CD8^-$; NKT cells: $CD3^+$, $CD19^-$, $CD4^-$, $CD8^-$, $CD16^+$, $CD56^+$; NK cells: $CD3^-$, $CD19^-$, $HLADR^-$, $CD16^+$, $CD56^+$; classical monocytes: $CD3^-$, $CD19^-$, $HLADR^+$ $CD16^-$, $CD14^+$; transitory monocytes: $CD3^-$, $CD19^-$, $HLADR^+$, $CD16^+$, $CD14^+$; unconventional monocytes: $CD3^-$, $CD19^-$, $HLADR^+$, $CD16^-$, $CD14^+$; conventional DCs (cDC): $CD3^-$, $CD19^-$, $HLADR^+$, $CD16^-$, $CD14^-$, $CD11c^+$, $CD123^{mid}$; plasmacytoid DCs (pDC): $CD3^-$, $CD19^-$, $HLADR^+$, $CD16^-$, $CD14^-$, $CD11c^-$, $CD123^+$. Percentage of (**B**) lymphoid and (**C**) myeloid cell populations over the kinetics. Left panels show the individual values for each patient. Right panels show the mean MFI ± SD from either the three 30-µg patients or four 100-µg patients. The statistical significance of the differences was determined by the paired Student's t-test relative to baseline. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

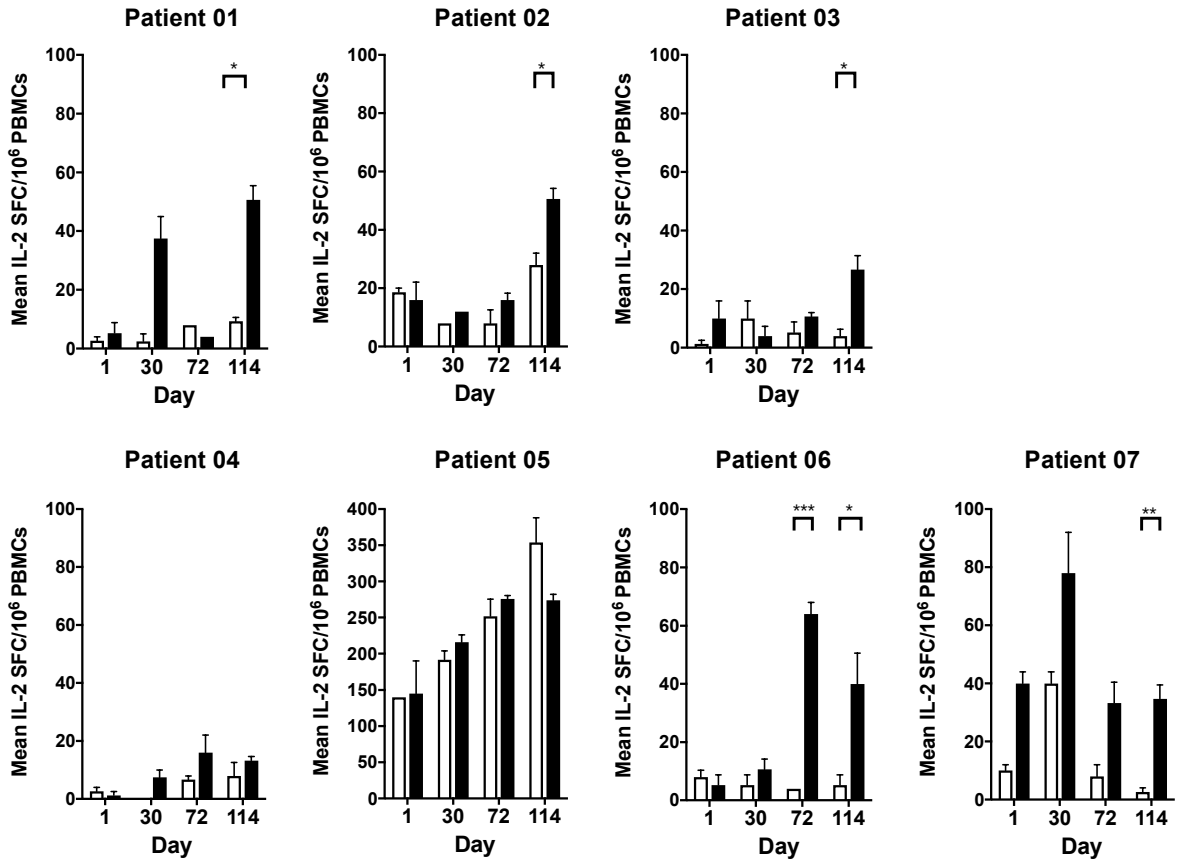


Figure S5. Immunization with the MAG-Tn3 vaccine induces the activation of a specific anti-TT T-cell response. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. PMBCs were harvested at various times after immunization and stimulated 18 h with either medium or the tetanus toxin ($TT_{830-844}$) peptide. IL-2 production was evaluated by ELISPOT and is expressed as the mean of spot-forming colonies (SFC) per 10_6 cells ± SEM of triplicates. The statistical significance of the differences was determined by the unpaired Student's t-test, comparing the SFC obtained with medium or the TT peptide for each timepoint. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

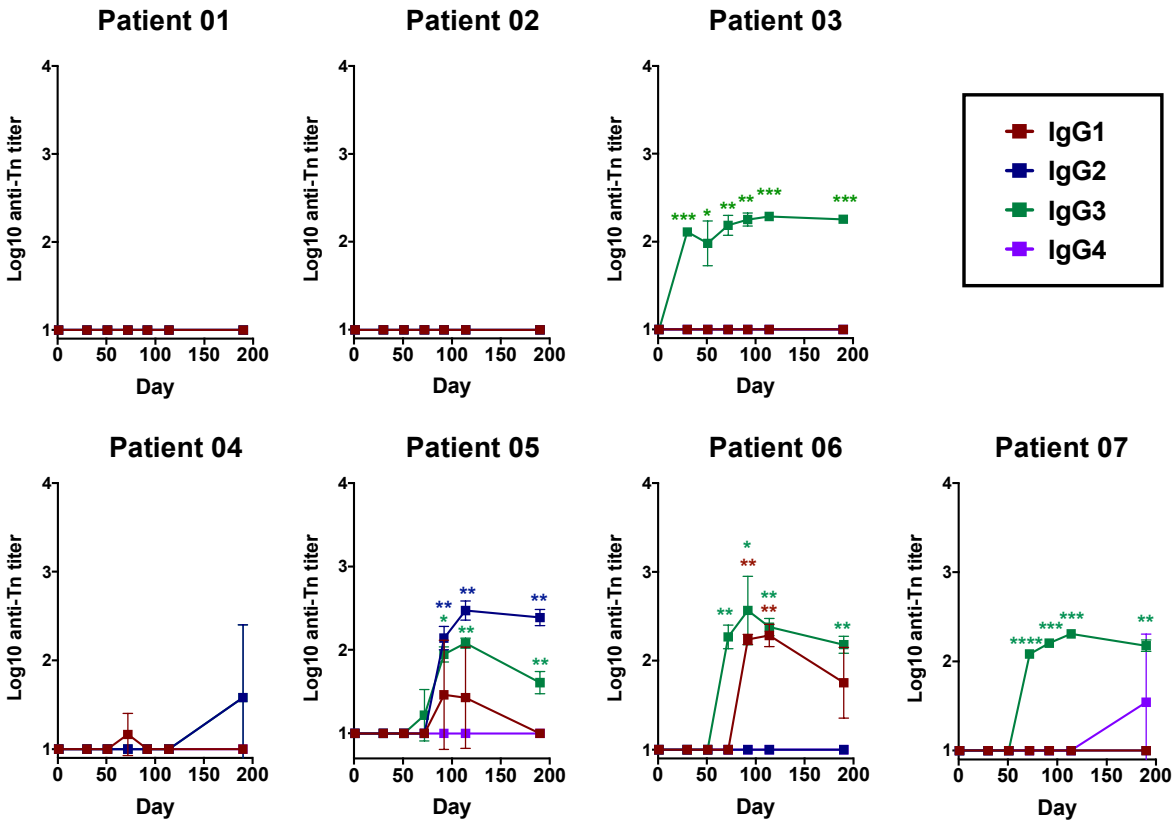


Figure S6. Immunization with the MAG-Tn3/AS15 vaccine induces an IgG3-oriented anti-Tn antibody response. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. Sera were collected at various times after immunization and tested for anti-Tn Ig1, IgG2, IgG3, and IgG4 specific antibody responses by ELISA. Antibody titers are expressed as the mean ± SD of the Log10 titer of quadruplicates from two independent experiments. The statistical significance of the differences was determined by the unpaired Student's t-test relative to baseline. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

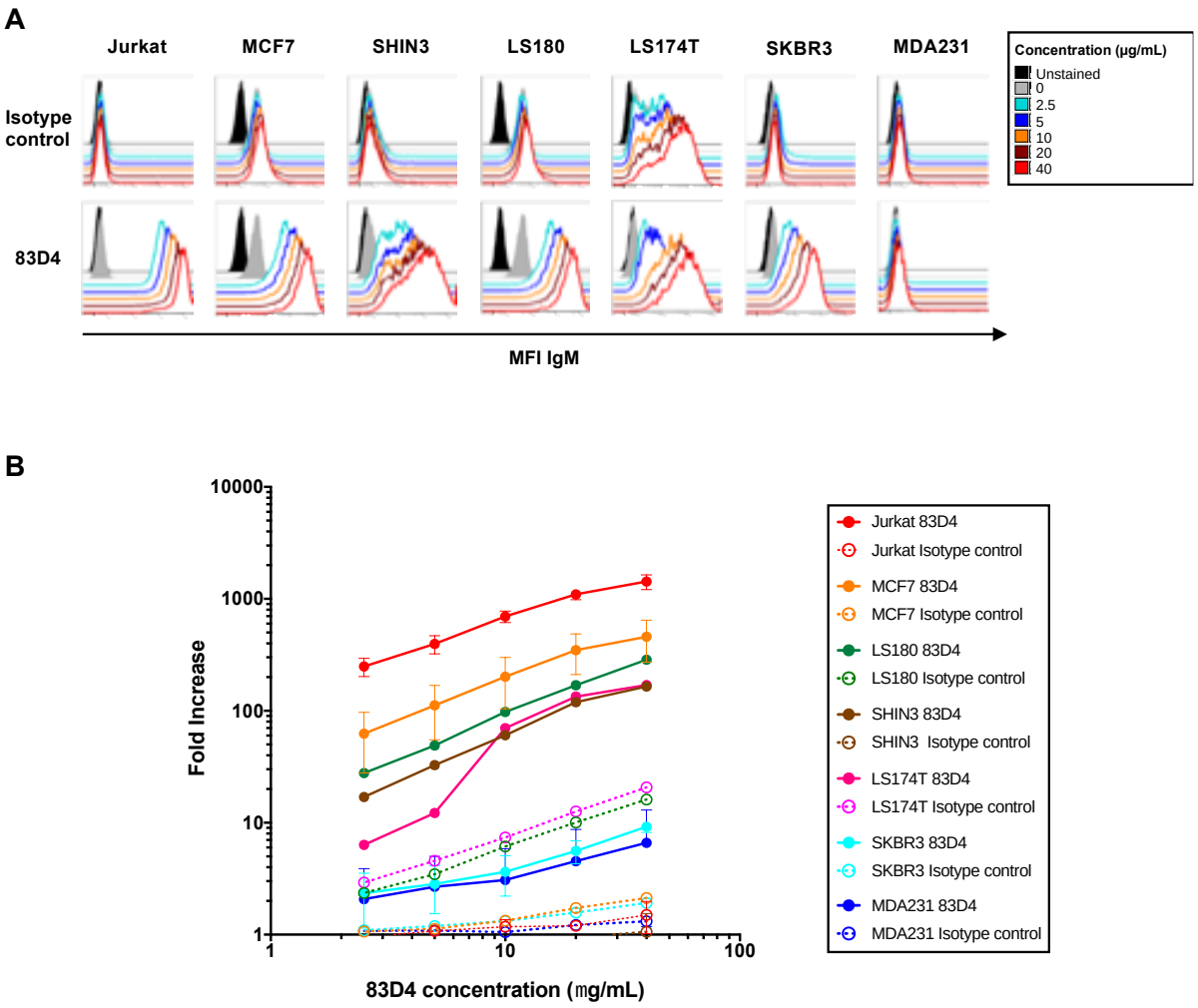


Figure S7. Analysis of the expression of the Tn antigen by the Jurkat, MCF7, SHIN3, LS180, LS174T, SKBR3, and MDA231 cell lines. The cells were incubated with various concentrations of the purified anti-Tn IgM mouse monoclonal antibody (83D4). Cell lines were then stained with anti-mouse IgM-FITC antibodies and analyzed by flow cytometry. The results are presented as histograms from one representative experiment (**A**) or as the fold increase (MFI of the sample in the presence of the antibody divided by the MFI in the absence of the antibody) (**B**) of MFI ± SD of duplicates or quadruplicates from two independent experiments.

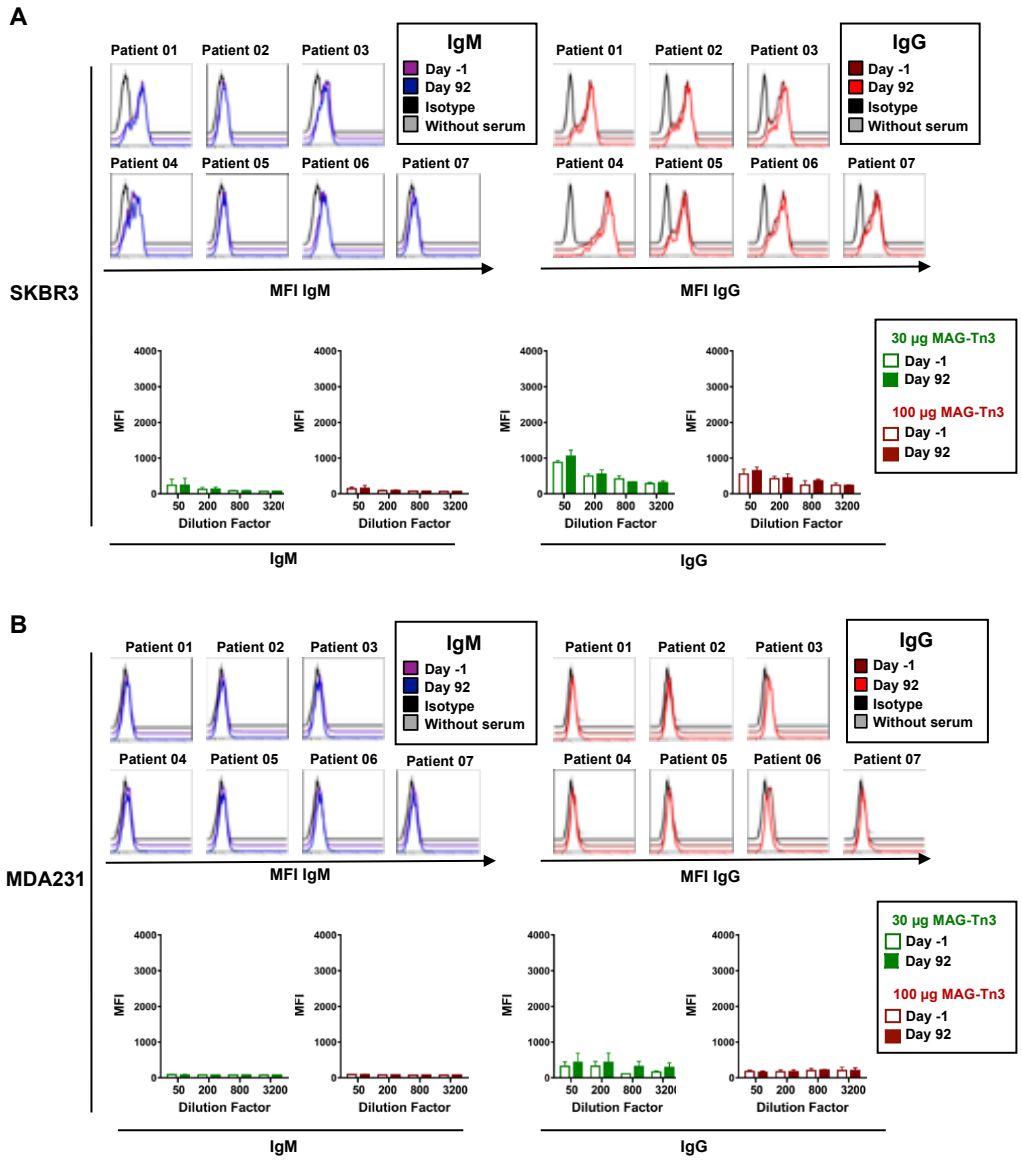


Figure S8. The serum of patients immunized with the MAG-Tn3/AS15 vaccine do not recognize Tn- human tumor cell lines. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. Pre- and post-immunized sera were analyzed for their ability to recognize (**A**) SKBR3 (Tn-) and (**B**) MDA231 (Tn-) cell lines. Cells were incubated with various dilutions of the sera and then stained with anti-human IgM-FITC or IgG-PE antibodies and analyzed by flow cytometry.

In **A** and **B**, the upper panels show histograms of one representative experiment showing the MFI for IgM and IgG expression at a 1/50 serum dilution for each patient. The lower panels show the mean MFI ± SD of two independent experiments from the sera of patients immunized either with 30 µg (left) or 100 µg (right) of MAG-Tn3. The statistical significance of the differences was determined by the paired Student's t-test, comparing the results obtained with sera before and after immunization. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

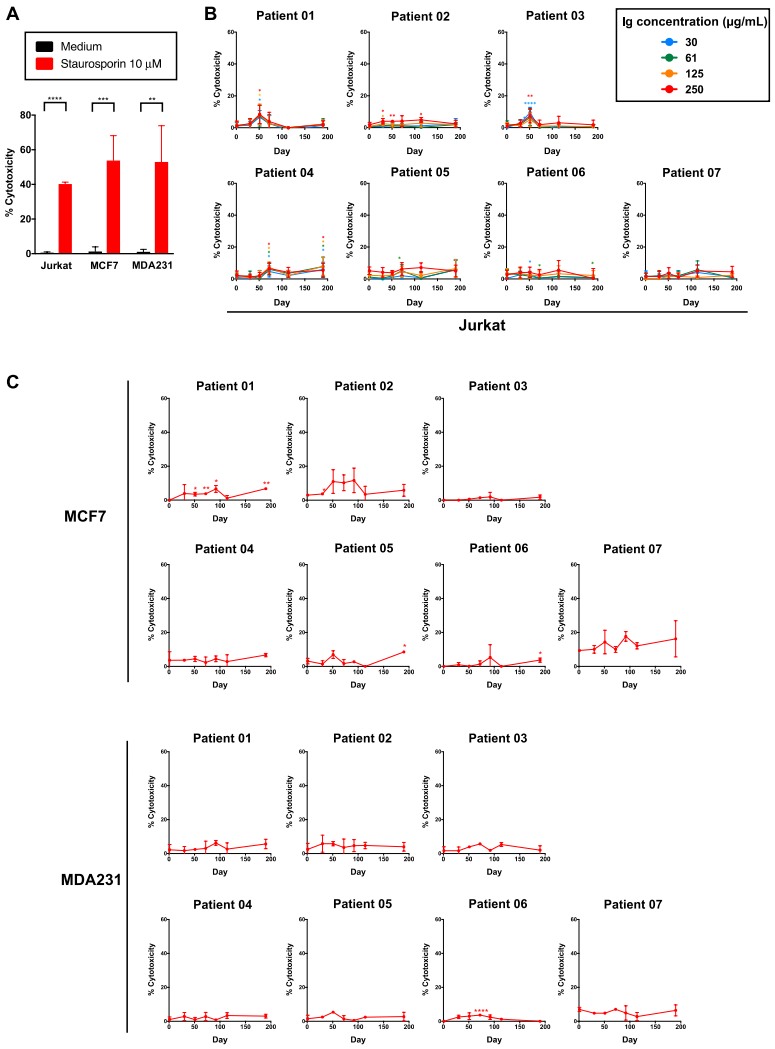


Figure S9. The antibodies induced in patients by immunization with the MAG-Tn3 vaccine do not directly kill Tn+ human tumor cells. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. Serums were collected at various times after immunization and the immunoglobulins purified and then incubated at various concentrations with the cell lines. After 5 h, cell viability was measured using the CellTiter Blue kit® (Promega). The percentage of direct cytotoxicity was calculated as described in Materials and Methods. (**A**) Direct cytotoxicity using 10 µM staurosporin, a pro-apoptotic molecule used as a positive control on Jurkat, MCF7, and MDA231 cell lines. The statistical significance of the differences was determined by the unpaired Student's t-test comparing the results obtained with staurosporin and the those obtained with medium. (**B**) Direct cytotoxicity of various concentrations of purified immunoglobulins on Jurkat cells (Tn+) and (**C**) MCF7 (Tn+) and MDA231 cells (Tn-) at 250 µg/mL. Data are expressed as the mean ± SD of the percentage of direct cytotoxicity from two to six replicates from two independent experiments. The statistical significance of the differences was determined by the unpaired Student's t-test relative to baseline. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

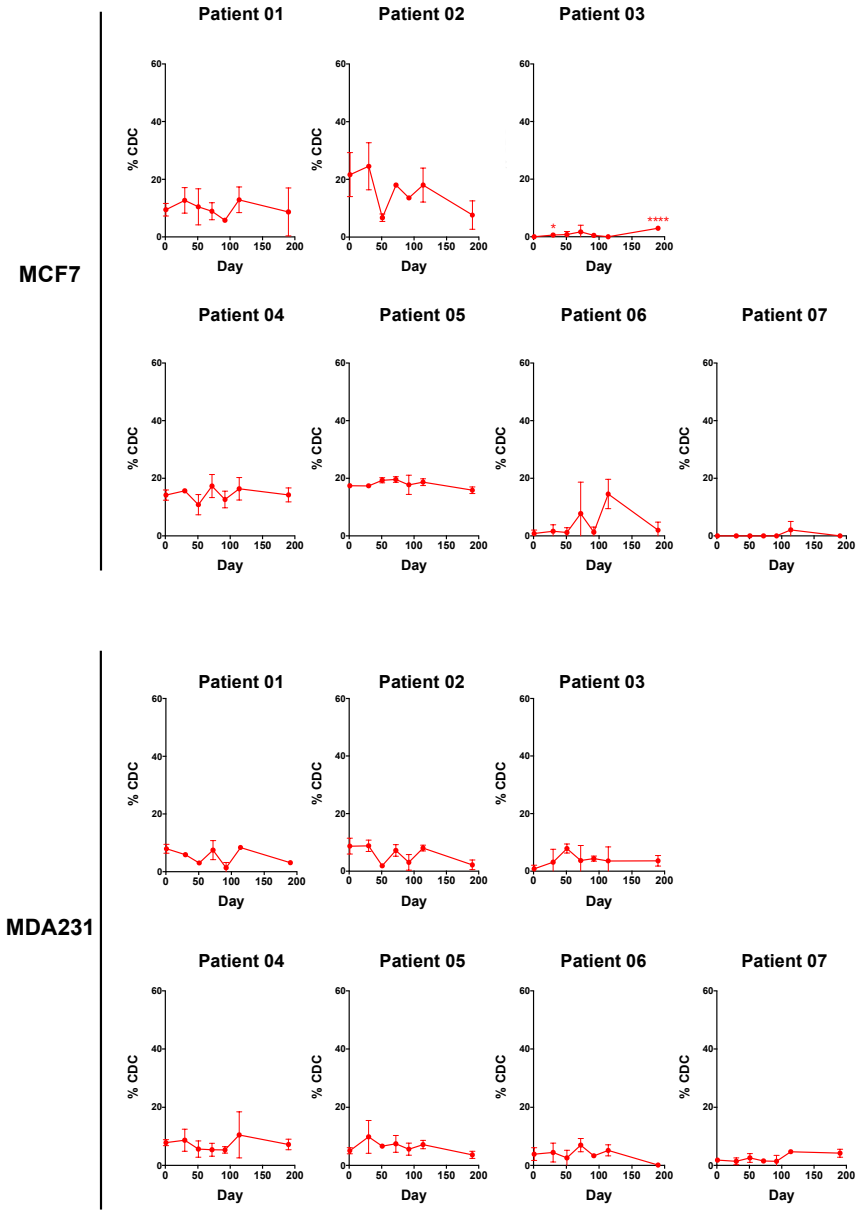


Figure S10. Lack of complement-dependent cytotoxicity on Tn+ MCF7 cells and Tn- MDA231 cells of anti-Tn antibodies induced in cancer patients by the MAG-Tn3 vaccine. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. Immunoglobulins purified from sera harvested at various times were incubated at 250 µg/mL with either Tn+ MCF7 or Tn- MDA231 cells, in the presence of complement. After 5 h of incubation, cell viability was measured using the Celltiter-Blue kit® (Promega). The percentage of CDC was calculated as detailed in Materials and Methods and the results are expressed as the mean ± SD of duplicates from two independent experiments. The statistical significance of the differences was determined by the unpaired Student's t-test relative to baseline. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

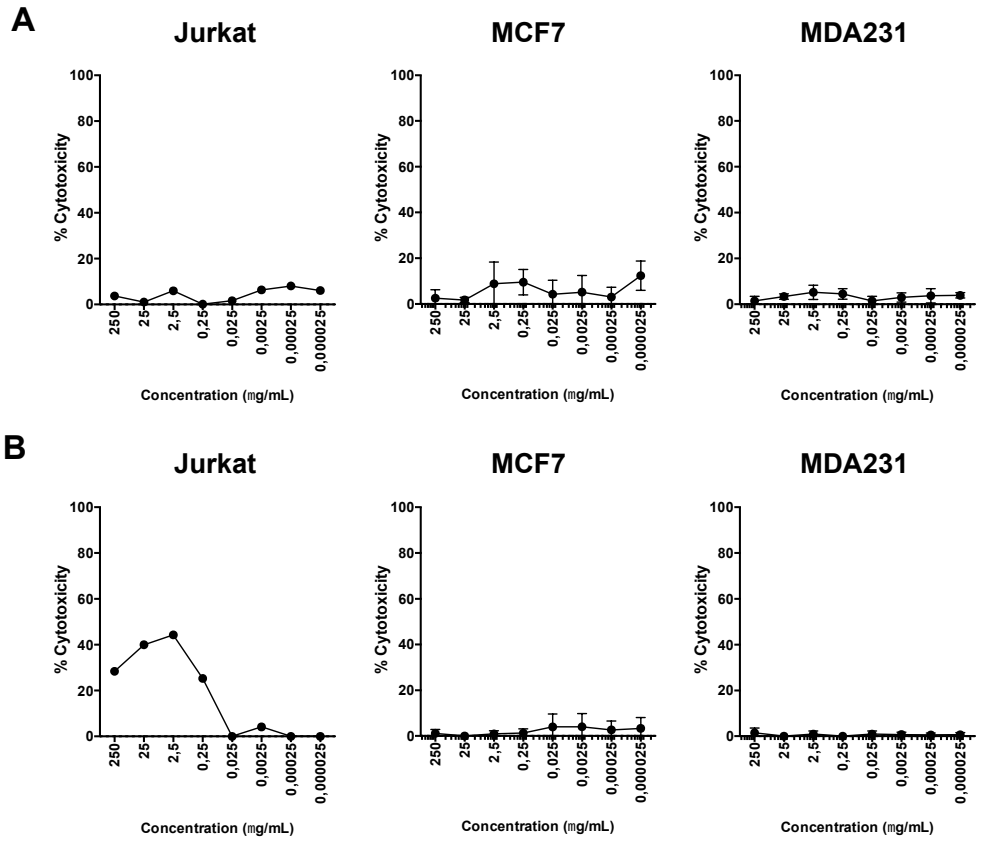


Fig. S11. The 8D4 anti-Tn monoclonal antibody induces the killing of Jurkat cells by complement-dependent cytotoxicity but not of MCF7 and MDA231 cells. The direct (**A**) and complement-dependent (**B**) cytotoxicity induced by the 8D4 anti-Tn monoclonal antibody at various concentrations on Jurkat, MCF7 and MDA231 cells were evaluated as described in the Figure 4 legend.

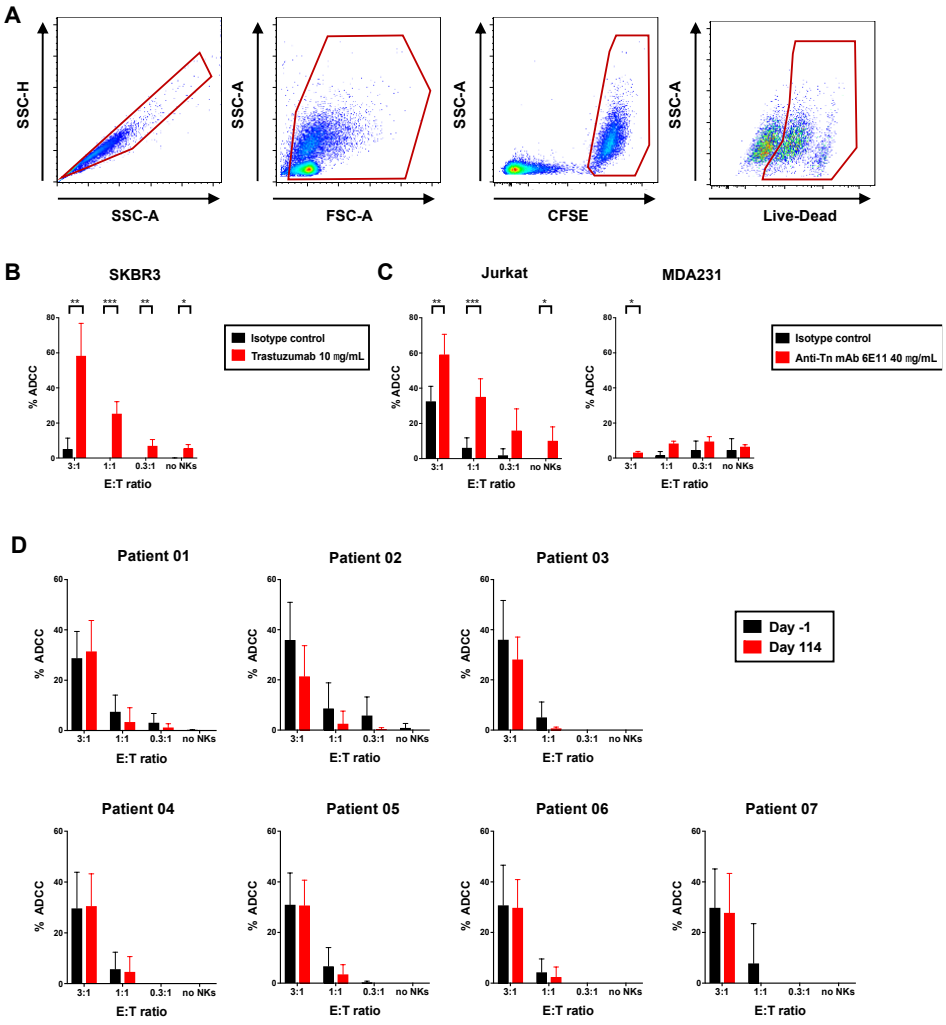


Figure S12. Lack of antibody-dependent cell cytotoxicity of anti-Tn antibodies induced in cancer patients by the MAG-Tn3 vaccine. Patients were immunized on days 0, 21, 42, 63, 84, and 105 with the MAG-Tn3 vaccine formulated with the immunostimulant AS15. Patients 01, 02, and 03 received intramuscular injections of 30 µg MAG-Tn3, whereas patients 04, 05, 06, and 07 received 100 µg MAG-Tn3. Immunoglobulins were purified from sera harvested at various times and their ADCC activity analyzed after 4 h of incubation of Ig with human primary CD56+ NK cells as effector cells and Jurkat, SKBR3, or MDA231 cells as target cells, at various E:T ratios. The percentage of ADCC was determined by flow cytometry (**A**) and calculated as detailed in Materials and Methods. (**B**) HER2+ SKBR3 cells were incubated with either 10 µg/mL of the humanized anti-HER2 trastuzumab monoclonal antibody or control isotype and NK cells at various E:T ratios. (**C**) Jurkat (Tn+) or MDA231 (Tn-) cells were incubated with either 40 µg/mL of the 6E11 anti-Tn murine monoclonal antibody or control isotype and NK cells at various E:T ratios. (**D**) Immunoglobulins purified from the sera of patients harvested at various times were incubated at 250 µg/mL with Tn+ Jurkat cells and NK cells at various E:T ratios. Data are expressed as the mean ± SD of quadruplicates from two independent experiments. The statistical significance of the differences was determined by the unpaired Student's t-test relative to baseline. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table S1. Patient hematology. Complete blood counts for leukocytes, lymphocytes, neutrophils, and monocytes for each patient at various times before and during immunization with the MAG-Tn3 vaccine formulated with AS15.

Patient	Cell population (10_6 cells/L)	Day						
		0	**30**	**51**	**72**	**92**	**114**	**190**
Patient 01	Leukocytes	4.4	3.4	3.7	3.8	4.3	3.7	4.4
	Lymphocytes	1.1	1.1	1.2	1.4	1.3	1.4	1.7
	Neutrophils	2.6	1.8	2	1.9	2.4	1.7	2.1
	Monocytes	0.4	0.3	0.3	0.3	0.4	0.4	0.4
Patient 02	Leukocytes	4.5	3.8	4.8	5.7	4.2	5.8	6.1
	Lymphocytes	1.4	1.3	1.6	2.4	1.4	2.1	1.7
	Neutrophils	2.5	2.0	2.8	2.7	2.3	3.0	3.4
	Monocytes	0.4	0.4	0.4	0.5	0.4	0.6	0.4
Patient 03	Leukocytes	7	5.5	5.4	5	5.2	5.8	6.3
	Lymphocytes	2.4	2	2.2	2	1.9	2.1	2.1
	Neutrophils	4.2	3.1	2.7	2.6	2.8	3.1	3.7
	Monocytes	0.4	0.4	0.4	0.3	0.4	0.4	0.4
Patient 04	Leukocytes	10.7	8.3	8.4	7.9	7.4	6.5	10.7
	Lymphocytes	1	1.1	1.4	0.9	1.1	1.1	1.3
	Neutrophils	8.4	6.1	6	6	5.4	4.5	8.5
	Monocytes	0.8	0.7	0.8	0.7	0.6	0.7	0.8
Patient 05	Leukocytes	5.1	4.6	3.8	5.9	3.5	4	3.5
	Lymphocytes	1.4	0.9	0.8	0.8	0.9	1	1
	Neutrophils	3	3.3	2.4	4.5	2	2.5	1.9
	Monocytes	0.5	0.3	0.3	0.4	0.4	0.4	0.4
Patient 06	Leukocytes	8.6	8.6	8.5	12.7	7.6	7.5	9.4
	Lymphocytes	2.5	2.1	2.7	2.9	2.2	2.4	2.2
	Neutrophils	5.3	5.7	5	8.7	4.7	4.4	6.4
	Monocytes	0.6	0.7	0.7	1.0	0.6	0.6	0.7
Patient 07	Leukocytes	4.1	5.9	3.7	4.1	3.7	4	4.5
	Lymphocytes	1.1	1.7	1.2	1.2	1.4	1.2	1.5
	Neutrophils	2.4	3.6	2.0	2.4	1.7	2.3	2.5
	Monocytes	0.3	0.5	0.4	0.3	0.5	0.3	0.4