Materials and Methods

Ethics Statement. The study has been approved by the Investigational Review Committee of University of Navarra Hospital (Comite’ de Investigacion Clinica de la Clinica Universitaria de Navarra), with written informed consent being on file. The clinical samples have been analyzed anonymously.

Animal care and welfare of animals. General procedures for animal care and housing were in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Standards incorporated in 9 CFR Part 3, 1991. Every effort was made to minimize, if not eliminate, pain and suffering in all animals in this study. All vertebrate animal experimentation work was approved by Touro University (Vallejo, CA, USA) under Institutional Animal Care and Use Committee (IACUC) protocol No. TUCA007AM02M-0609 or the University of Navarra (Pamplona, Spain) under IACUC protocol No. 028/09. While adhering to animal care and welfare guidelines, the Study Director made every effort to protect the scientific validity of the study.

Biopsy Samples. Twenty biopsy samples obtained from the Laboratory of Immunotherapy of the Center for Applied Medical Research (University of Navarra, Spain) were used as the starting material for cloning the variable regions of tumor immunoglobulins (Ig). In 6/20 cases, repeated attempts to rescue the Id through hybridomas had systematically failed and, as such, those patients never received an Id vaccine. In addition, two mouse lymphoma cell lines, 38C13 [32] and A20 [33], kindly provided by Shoshana Levy and Maurizio Bendandi, respectively, have been used in this study.

Cloning and Identification of Id Variable Regions. The protocol from Osterroth et al. [18] was modified using four constant region specific primers per immunoglobulin class, respectively, in a single round of anchored PCR amplification using highly processive KOD DNA polymerase (Merck, Darmstadt, Germany). PCR amplification was followed by specific cloning of Ig fragments and sequencing of 96 resulting clones per immunoglobulin class. When the Id sequence has been determined unequivocally, specific primers were designed to amplify the variable regions of the heavy and light chains (without native leader sequences). The variable regions of Id heavy and light chains were cloned in TMV (tobacco mosaic virus) and PVX (potato virus X) based magnICON® vectors using “Golden Gate”
cloning [31]. Each cloning vector contains two BsaI restriction sites located between a plant signal peptide and a plant codon usage-optimized IgG1, kappa or lambda constant region, respectively. The vectors also contain a lacZα gene for blue-white selection to facilitate screening of recombinant clones. For cloning, vector and insert are digested with BsaI, ligated, transformed in E. coli, and plated on LB medium containing X-Gal and antibiotics. DNA minipreps of four colonies were initially screened by restriction digest and two clones per Ig chain with correct restriction patterns were confirmed by sequencing. DNA was then electroporated into Agrobacterium (industrial strain ICF 320, ΔcysKα, ΔcysKβ, ΔthiG derivative of Agrobacterium tumefaciens C58).

**Id Expression, Purification and Conjugation with KLH.** A non-competing viral vectors system was used to express the Ig in Nicotiana benthamiana leaves [15]. N. benthamiana accession used was an industrial line developed and characterized by Bayer Bioscience. Agro-infiltrated plants were incubated in the greenhouse for 7 days, then green biomass was harvested, Ig extracted and crude extract analyzed using non-reducing polyacrylamide gel electrophoresis as described earlier [15].

For Ig production, both selected Agrobacterium strains harboring PVX and TMV vectors were grown separately in LB media with soya peptone (Duchefa Biochemie, Haarlem, The Netherlands) replacing tryptone. Bacterial cultures were grown at 28°C until the OD₆₀₀ reaches 3 to 4. Infiltration solution was prepared by mixing and diluting both bacterial cultures in infiltration buffer (10 mM MES, pH 5.5, 10 mM MgSO₄) to a defined cell concentration (equivalent to a 500-fold dilution of a culture with OD₆₀₀ of 1.0). Nicotiana benthamiana (‘ICON’ accession) plants grown under controlled and standardized conditions were vacuum-infiltrated with the agrobacterial suspension (mixture of two agrobacterial strains harbouring TMV and PVX vectors) and then kept in the growth chambers for 7 days for Ig expression and accumulation. Plant biomass was then harvested, mixed with two volumes (w/v) of pre-chilled to +4°C extraction buffer (200 mM Tris-HCl, pH 7.5, 5 mM EDTA) and homogenized in a blender. The pH of the homogenate was lowered to <5.1 for removal host cell proteins including Rubisco. Subsequently the pH of the homogenate was adjusted to 8.5 with NaOH and then the crude extract was clarified by dead-end filtration.

The Ig from extract was purified using Protein A affinity chromatography [25]. All chromatography steps are carried out at room temperature using a GE Healthcare ÄKTA
Purifier Chromatography System. Column-bound protein was eluted with a low pH buffer via a peak-based fractionation and adjusted to a neutral pH. The Ig-containing eluate was further purified by membrane adsorption chromatography on an anion exchange membrane adsorber. Before the conjugation step, the Ig solution was filtered using 0.2 µm filter to reduce bioburden. Conjugation of Ig with keyhole limpet hemocyanin (KLH) was performed using glutaraldehyde as described [3].

**Characterization and analysis of Ig and conjugate.** The concentration of final product was determined by using the BCA assay (Pierce, Rockford, IL). For the characterization of Ig intermediates, purified immunoglobulins were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie-staining and by capillary gel electrophoresis (Agilent Bioanalyzer 2100, Santa Clara, US). Capillary gel electrophoresis was performed using a lab-on-a-chip format in reduced and non-reduced mode. The electropherograms were integrated and purity was calculated. Additionally, a tryptic peptide mapping method has been used. Light and heavy chain were separated by reduced SDS PAGE, digested and analyzed by MALDI-MS. N-terminal sequencing of the light and heavy chain has been carried out by a contract facility. The first 5 to 8 amino acid residues were determined and compared to the expected N-terminal sequence. Glycosylation of immunoglobulins was analyzed by immunoblotting using a mixture of anti-fucose and anti-xylose antibodies (kindly provided by Bayer Bioscience) for detection. For Ig quantification by ELISA, the wells of a microtiter plate was coated with polyclonal anti-human IgG, gamma-chain-specific capture antibody (Antikörper online, Germany). After washing and blocking, serial dilutions of crude leaf extracts, buffer control and standards (human kappa IgG1, human lambda IgG1; Sigma-Aldrich, Germany) were applied to the wells and incubated. For detection anti-human kappa light chain alkaline phosphatase conjugate or anti-human lambda light chain alkaline phosphatase conjugate (Sigma-Aldrich, Germany) were used.

**Cross-reactivity of Ids.** Depending on the availability of reagents, we tested possible cross-reactivity of hybridoma- and plant-derived Igs with the sera of a few patients who had been previously vaccinated with the former. Tumor-derived Ids were rescued by hybridoma-based methodology as described [19, 20] and administered as recently outlined [30]. Cross-reactivity of hybridoma- and magnICON®-derived Ids was assessed by standard ELISA as
described [3, 4, 21]. In brief, plant-made Ig was coupled on ELISA plate and serial dilutions of patient pre- and post vaccine sera were added. For detection, anti-human light chain (kappa when the patient light chain was lambda, or lambda, when the patient light chain was kappa) was used. An anti-Id humoral response was considered specific provided that the following conditions were both met: a) the optical density (OD) ratio between post- and pre-vaccine sera was at least 4-fold in one dilution and 2-fold in another dilution, or at least 3-fold in two dilutions, or at least 2-fold in three dilutions; b) the OD ratio above, compared with the corresponding OD ratio of an irrelevant, isotype-matched Id control was also at least 4-fold in one dilution and 2-fold in another dilution, or at least 3-fold in two dilutions, or at least 2-fold in three dilutions [34].

**Animal Vaccination and Analysis.** Purified plant-produced 38C13 IgG1-KLH (10 µg or 50 µg/100 µl) was administered to female C3H/HeN mice (supplied by Charles River, Hollister, CA) subcutaneously (s.c.) in the rear flank at two-week intervals for a total of two vaccinations. The “Gold Standard” 38C13 IgM-KLH (50 µg) was administered s.c concurrently with the ICON IgG1-KLH vaccinations. Mice receiving vaccine were co-administered 10⁴ U/mouse (~2 µg/100 µl) of recombinant, murine-specific GM-CSF adjuvant once on the day of vaccination and for three consecutive days thereafter. Vaccinations with PBS (vehicle) or KLH+GM-CSF (carrier protein/adjuvant) alone were used as negative controls. Vaccinated animals were bled through the tail vein 10 days after the first and second vaccinations, and individual anti-38C13 animal responses were measured in serum by anti-38C13 scFv ELISA. ELISA plates were coated with 5 mg/ml 38C13 scFv in carbonate buffer, washed, and then incubated with a dilution series of serum. Total IgG levels were quantitated against purified S1C5 isotype-specific mouse anti-38C13 antibody, using an anti-mouse pan IgG horse radish peroxidase-conjugated secondary antibody for detection. Ig isotype analysis was performed on pooled sera from each vaccine group after the first and second vaccination.

Purified plant-produced A20 IgG1-KLH (50 µg/100 µl) was administered to female BALB/c mice (supplied by Charles River, Hollister, CA) subcutaneously (s.c.) in the rear flank at one-week intervals for a total of four vaccinations. The native A20 IgG2a-KLH (50 µg) was administered s.c. concurrently with the ICON IgG1-KLH vaccinations. Mice receiving vaccine were co-administered 10⁴ U/mouse (~2 µg/100 µl) of recombinant, murine-specific GM-CSF adjuvant once on the day of vaccination and for three consecutive days thereafter.
Vaccinations with PBS (vehicle) or KLH+GM-CSF (carrier protein/adjuvant) alone were used as negative controls.

**Tumor Challenge and Analysis.** With respect to the 38C13 model, two weeks after the last vaccination, animals were challenged with 1500 tumor cells that were grown and prepared for subcutaneous administration as follows. Approximately $10^6$ 38C13 tumor cells were thawed, washed, resuspended in 10 ml RPMI supplemented with L-glutamine, 10% FCS, 1x penicillin/streptomycin, 50 µM 2-mercaptoethanol, and grown at 5% CO$_2$ in a 37°C humidified incubator. Two days later, cells were split 1:10 into fresh media, and used the following day for tumor challenge. Cells were harvested, washed twice in HBSS to remove FCS, counted, and resuspended in HBSS at $1.5 \times 10^4$ cells/ml; 0.1ml was administered subcutaneous, yielding a total dose of 1500 cells per animal. After eight days animals were checked for visible subcutaneous tumors. After tumor appearance, tumor area (length x width) was measured by digital Vernier calipers, and recorded. When tumor area reached 2.0 cm, animals were euthanized according to IACUC protocol. Kaplan-Meier analysis was used to plot survival curves, and log-rank analysis was used to determine statistically significant differences in survival.

With respect to the A20 model, ten days after the last vaccination, animals were challenged with 500,000 tumor cells that were grown and prepared for subcutaneous administration as follows. Approximately $10^6$ A20 tumor cells were thawed, washed, resuspended in 10 ml RPMI supplemented with L-glutamine, 10% FCS, 1x penicillin/streptomycin, 50 µM 2-mercaptoethanol, and grown at 5% CO$_2$ in a 37°C humidified incubator. Two days later, cells were split 1:10 into fresh media, and used the following day for tumor challenge. Cells were harvested, washed twice in HBSS to remove FCS, counted, and resuspended in HBSS at $2.5 \times 10^6$ cells/ml; 0.2ml was administered subcutaneous, yielding a total dose of 500,000 cells per animal. After eight days animals were checked for visible subcutaneous tumors. After tumor appearance, tumor area (length x width) was measured by digital Vernier calipers, and recorded. When tumor area reached 2.0 cm, animals were euthanized according to IACUC protocol. Kaplan-Meier analysis was used to plot survival curves, and log-rank analysis was used to determine statistically significant differences in survival.