Antigen-specific *In Vitro* Expansion of Functional Redirected NY-ESO-1-specific Human CD8+ T-Cells in a Cell-free System

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Abstract. *Background: Tumors can be targeted by the adoptive transfer of chimeric antigen receptor (CAR) redirected T-cells. Antigen-specific expansion protocols are needed to generate large quantities of redirected T-cells. We aimed to establish a protocol to expand functional active NY-ESO-1-specific redirected human CD8+ T-cells. Materials and Methods: The anti-idiotypic Fab antibody A4 with specificity for HLA-A*0201/NY-ESO-1$_{157-165}$ was tested by competition assays using a HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer. HLA-A*0201/NY-ESO-1$_{157-165}$ redirected T-cells were generated, expanded and tested for CAR expression, cytokine release, in vitro cytolysis and protection against xenografted HLA-A*0201/NY-ESO-1$_{157-165}$–positive multiple myeloma cells. Results: A4 demonstrated antigen-specific binding to HLA-A*0201/NY-ESO-1$_{157-165}$ redirected T-cells. Expansion with A4 resulted in 98% of HLA-A*0201/NY-ESO-1$_{157-165}$ redirected T-cells. A4 induced strong proliferation, resulting in a 300-fold increase of redirected T-cells. After expansion protocols, redirected T-cells secreted Interleukin-2, (IL-2), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) and lysed target cells in vitro and were protective in vivo. Conclusion: A4 expanded HLA-A*0201/NY-ESO-1$_{157-165}$ redirected T-cells with preservation of antigen-specific function.*

It is now widely accepted that T-cells play an important role in controlling tumor growth (1) and tumor-specific T-cells can be detected at variable numbers in individual tumor samples (2-5). However, previous studies have shown that these tumor-infiltrating lymphocytes (TILs) are often functionally impaired due to a plethora of non-redundant mechanisms, including inhibitory cytokines, regulatory T-cells (Tregs) and myeloid-derived suppressor cells (6, 7). There is increasing evidence that the adoptive transfer of autologous, *in vitro*-activated and -expanded tumor-specific T-cells may circumvent these problems and thus may represent an attractive therapeutic option. Indeed, results from multiple clinical trials show promising objective responses in patients with cancer (8-10). The use of autologous T-cells that are retrovirally transduced to express a relevant T-cell receptor (TCR) or chimeric antigen receptor (CAR) is presumably more effective than the use of endogenous tumor-specific T-cells because the latter may be functionally compromised (11) or carry T-cell receptors with insufficient affinity. TCR-grafted T-cells recognize major histocompatibility complex (MHC) class I/peptide complexes, whereas CAR-grafted T-cells recognize MHC class I/peptide complexes or surface proteins (12, 13). Both receptors contain transmembrane and intracellular TCR signaling domains (14, 15).

Based on available data, the adoptive transfer of a large number of redirected T-cells must be transferred for clinical efficacy. Therefore, strategies allowing for large-scale expansion of functionally intact redirected T-cells under good manufacturing practice (GMP) conditions need to be developed. The currently available methods all rely on stimulation with antigen-presenting cells pulsed with specific peptide on artificial antigen-presenting cells (16, 17) or on polyclonal stimulation using either lectins or antibodies against CD3 and CD28 (18, 19). These methods have potential disadvantages, for example, expansion of T-cells using antigen-presenting cells is technically challenging, depends on the use of third-party cells, more difficult to standardize and, therefore, not a straightforward GMP approach. Polyclonal stimulation results in large numbers of T-cells independent of their specificity, which bears the risk of off-target toxicity.

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We have generated an anti-NY-ESO-1 CAR that specifically targets the HLA-A*0201/NY-ESO-$1_{157-165}$ peptide complex (20, 21). To expand HLA-A*0201/NY-ESO-$1_{157-165}$ redirected T-cells in an antigen-specific manner but without feeder cells, we generated anti-idiotypic Fab molecules specific for the anti-NY-ESO-1 CAR. Here, we describe the characterization of these anti-idiotypic antibodies and their use for expanding redirected T- cells expressing NY-ESO-1 peptide-specific CARs.

Materials and Methods

Cell lines. 293 Cells expressing the SV40 large T-antigen (293T cells) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HLA-A*0201-positive, transporter associated with antigen processing (TAP)-deficient T2 cells stably transfected with minigenes (1B: NY-ESO-$1_{157-165}$ and 1C: NY-ESO-$1_{155–163}$) were obtained from J. Cebon, Ludwig Institute for Cancer Research (LICR), Melbourne, Australia and have been described previously (22). U266 is an HLA-A*0201, NY-ESO-$1_{157-165}$–positive myeloma cell line expressing human immunoglobulin E (IgE). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen, Karlsruhe, Germany) (R10 medium); 50 μg/ml hygromycin B were added to cultures of transfected cells.

Expression and purification of anti-idiotypic Fab antibodies. *Escherichia coli* (*E. coli*) TG-1 (Zymo Research, Irvine, CA, USA) cultures expressing anti-idiotypic Fab antibodies were diluted at 1:100 ratio with fresh 2xYT broth and grown overnight (18 h), containing 100 μg/ml ampicillin and 0.1% glucose and grown at 37˚C. Cells were induced with isopropyl-β-D-1-thiogalactopy-ranoside (IPTG) when an OD_{600} value of 0.8-1 was reached and further grown at 30 ˚C for 4 h. Cells were then centrifuged at 4,000 rpm (3750 × *g*) for 15 min and periplasmic proteins were isolated by osmotic shock (23). His-tag bearing Fabs were purified using Talon metal affinity resin (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions and the purity of Fabs was assessed by 12% sodium dodecyl sulfate (SDS) gel electrophoresis.

*Binding of anti-idiotypic Fab antibodies to anti-NY-ESO-$1_{157-165}$/HLA-A*0201*. A4 anti-idiotypic Fabs were coated on the surface of 96-well enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Langenselbold, Germany) at a concentration of 0.01 μg/μl in carbonate buffer (pH 9.5) at 37˚C for 3-4 h. Unbound Fabs were removed by washing with phosphate buffer saline (PBS) (pH 7.4). Wells were subsequently blocked with PBS-containing 10% FCS for 1 h at room temperature (RT). Anti-NY-ESO-$1_{157-165}$/HLA-A*0201 or control antibodies to human IgG (1 ng/μl) were added to the anti-idiotypic antibody coated wells and incubated for 1 h at RT. Plates were washed three times with PBS-containing 0.05% Tween-20 to remove unbound antibodies. Binding of anti-NY-ESO-$1_{157-165}$/HLA-A*0201 was detected with horseradish peroxidase (HRP)-labeled human Fc-specific antibody (Immuno Research, Newmarket, Suffolk, UK), according to the manufacturer's instructions.

Binding of anti-idiotypic Fab antibodies to redirected T-cells expressing anti-NY-ESO-1 CAR. 293T cells were transfected with the anti-NY-ESO-1 CARs, as described previously (21). After 24 h, 10^5 anti-NY-ESO-1 CAR-grafted 293T cells were washed twice with PBS (pH 7.5) containing 2 mM EDTA and 0.05% FCS (FACS buffer). Cells were resuspended in 0.01 μg/μl anti-idiotypic Fab-containing FACS buffer and incubated for 30 min at 4˚C. Cells were washed with FACS buffer and His-tag bearing Fabs were incubated with mouse monoclonal antibody to His-tag (Qiagen, Hombrechtikon, Switzerland) for 30 min. Finally, cells were stained with phycoerythrin (PE)-labeled antibody to mouse IgG1 (Southern Biotech, Birmingham, AL, USA). Binding of anti-idiotypic Fabs and control antibodies was analyzed by FACScan (BD Bioscience, San Diego, CA, USA). Data were analyzed using FlowJo software (Tree Star, Asland, OR, USA).

Competition assay of binding of anti-idiotypic Fab molecules. A total of 10^5 anti-NY-ESO-1 CAR redirected CD8+ T-cells were washed twice with FACS buffer and incubated with different concentrations of anti-idiotypic Fab A4 (1, 0.25, 0.03 μg/μl) for 15 min at RT. Irrelevant Fab served as a control for anti-idiotypic Fab. Ten microliters of PE-labeled HLA-A*02:01/NY-ESO-$1_{157-165}$ tetramer (2 ng/μl) were added to each sample. Tetramer alone served as a negative control. Tubes were incubated for 5 min at RT. Cells were then washed with FACS buffer and inhibition of tetramer binding was determined by measuring fluorescent intensity using a FACScan flow cytometer (BD Bioscience, San Diego, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Generation of anti-NY-ESO-1 redirected CD8+ T-cells. Redirected T-cells were generated as described previously (21). In short, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by density centrifugation using Ficoll gradient. CD8+ T-cells were negatively selected by magnetic bead sorting using a CD8+ T-cell isolation kit (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Anti-HLA-A*0201/NY-ESO-$1_{157-165}$ CAR contains a CD28 and CD3ζ domain and is termed anti-NY-ESO-1 CAR hereafter. The BW431/26-CD28/CD3ζ CAR construct that directly recognizes the carcinoembryonic antigen (CEA) served as control (24) and is referred to as anti-CEA CAR. The retroviral transduction of CD8+ T-cells with recombinant receptors was performed by co-culturing the polyclonally activated CD8+ T-cells with transiently transfected 293T cells as described (21). After 24 h of co-cultivation, expression of CARs was monitored by flow cytometry using phycoerythrin (PE)-labeled anti-human IgG1, HLA-A*0201/NY-ESO-$1_{157-165}$ tetramer labeled with PE or fluorescein isothiocyanate (FITC)-conjugated anti-human-CD8 (Biolegend, San Diego, CA, USA).

Antibodies and cytokines. Anti-human IgG-PE, anti-mouse IgG1-PE (Southern Biotech, AL, USA), anti-human CD8-FITC, anti-human CD8-PE-Texas Red, anti-human interferon gamma (IFNγ)-FITC, anti-human tumor necrosis factor alpha (TNFα)-PE-Cy7 and anti-human Interleukin-2 (IL2)-APC (Biolegend, San Diego, CA, USA) were used for flow cytometry. The HLA-A2-restricted NY-ESO-$1_{157-165}$-specific PE-conjugated tetramer for surface staining of anti-NY-ESO-1 CAR redirected CD8+ T-cells was kindly provided by Dr. Luescher (LICR, Lausanne, Switzerland). For T-cell activation, monoclonal antibodies to human CD3 (OKT3) and human CD28 (15E8) were purchased from eBioscience (San Diego, CA, USA). Recombinant human IL-2 was obtained from Immunotools (Friesoythe, Germany). All monoclonal antibodies and tetramers were used according to the manufacturer's instruction.

Intracellular cytokine staining (ICS). A total of 2×10^5 anti-NY-ESO-1 CAR redirected CD8+ T-cells were incubated in 200 μl of R10 medium with either 2×10^5 T2-1B cells, 2×10^5 T2-1C cells or with medium alone (control) in the presence of 5 μg/ml Brefeldin A and 5 μg/ml monensin at 37˚C for 5 h. Cells were surface-stained with monoclonal antibodies to human CD8-PE-Texas Red and human IgG-PE (15 min at 4˚C). After surface staining, cells were washed with FACS buffer (FB; PBS plus 2% FCS plus 40 mM EDTA and 0.05% NaN3), fixed with 2% paraformaldehyde and permeabilized with permeabilization buffer (PB; FACS buffer plus 0.1% saponin). Cells were stained for intracellular IFNγ, TNFα and IL-2 with specific monoclonal antibodies (15 min at 4˚C). Samples were measured with a CyAn ADP9 flow cytometer (Beckman Coulter, Brea, CA, USA) and results were analyzed using FlowJo analysis software.

Activation of anti-NY-ESO-1 CAR redirected CD8+ T-cells with anti-idiotype or with NY-ESO-$1_{157-165}$ peptide pulsed HLA-A2 dimer. Anti-idiotypic Fab antibody or recombinant HLA-A2 dimer (BD Bioscience, San Diego, CA) (2 to 200 nM) were coated on 96-well cell culture plates at 37˚C for 3 h or at 4˚C overnight. Plates were washed with PBS to remove unbound molecules before adding 100 μl of 10 μM NY-ESO-$1_{157-165}$ peptide to the recombinant HLA-A2-coated wells that were subsequently incubated at 37˚C for 3 h. Plates were then washed with PBS and 2×10^4 and 5×10^3 (for HLA-A2 and A4 comparison) anti-NY-ESO-1 CAR redirected CD8+ T-cells were added to each well. After 24 h of stimulation, secretion of IFNγ was measured using an IFNγ ELISA kit (BD OptEIA™ , San Diego, CA, USA) according to the manufacturer's instructions.

Antigen-dependent expansion of anti-NY-ESO-1 CAR redirected CD8+ T-cells. Tissue culture flasks of 25 cm^2 were coated with 2 μg/ml of specific anti-idiotypic Fab antibodies to NY-ESO-1 CAR or control Fab (irrelevant Fab) molecules and incubated at 37˚C for 3-4 h. Subsequently, 10^6 anti-NY-ESO-1 CAR-positive redirected CD8+ T-cells were added to the flasks in 5 ml of RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (R10 medium) and 50 IU/ml of human recombinant IL-2. The medium was replaced with fresh R10 medium containing human recombinant IL-2 50 IU/ml) after 24 h of stimulation. For T2-1B-mediated activation, 2.5×10^5 irradiated (γ-irradiation with 50 Gy) stimulator T2-1B cells were co-cultured as described above at a ratio of 4 to 1 (effector to target). Redirected T-cells were stimulated repetitively with irradiated T2-1B cells or anti-idiotypic antibody every eight days. An increase in receptor-positive cells was identified by surface staining with antibody to human IgG and the number of cells was determined by counting viable cells on a weekly basis.

Colorimetric analysis of cell cytotoxicity. CD8+ T-cells were co-cultivated in 96-well round bottom microtiter plates at different numbers (ranging from 2.5×10^2-2×10^4 CAR-positive T-cells per well) with 10^4 HLA-A2/NY-ESO-$1_{157-165}$-positive cells (T2-1B) or control cells in 200 μl of R10 medium. After 24 h XTT (sodium 3'-(1-phenylamincarbonyl)-3,4-tetrazolium–(4-methoxy-6-nitro)-benzene-sulfonic-acid-hydrate) reagent (Cell Proliferation Kit II, Roche Diagnostics, Rotkreuz, Switzerland) was added to the cells which were then incubated at 37˚C for 30-90 min. Reduction of XTT to formazan by viable tumor cells was colorimetrically monitored. Maximal reduction of XTT was determined as the mean of three wells containing target cells only, and the background as the mean of three wells containing R10 medium alone. The non-specific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells at the same number as the corresponding experimental wells (24).

Xenograft model. Non-obese Diabetic-Severe Combined Immunodeficiency (NOD-SCID) γc (null) (NSG) mice (NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}*/SzJ) were bred and maintained under specific pathogen-free conditions in-house. Sex-matched NSG mice were randomized, and divided into three groups (each of 5 mice) and were injected subcutaneously with 10^7 multiple myeloma U266 tumor cells with or without 10^7 of expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells. Tumor growth was assessed by measuring serum IgE levels every week and tumor size was measured with the aid of calipers in two perpendicular dimensions. The tumor volume was calculated using the formula: tumor size (mm^2)=(tumor length × width).

Statistical analysis. The prism software was used for the analysis of statistical significance. Data was presented as SEM and difference between two groups was analyzed by applying the unpaired student's *t*-test.

Results

*Characterization of anti-idiotypic Fab molecules recognizing HLA-A*0201/NY-ESO-$1_{157-165}$.* Anti-idiotypic antibodies to the NY-ESO-$1_{157-165}$/HLA-A*0201 antibody were selected from a phage display library, as described previously (23). Two candidate Fabs (H6 and A4) were characterized in more detail and Fab A4 was chosen for further experiments since its binding and expression properties were superior (data not shown). Fab A4 was produced in *E. coli* with an expected size of 25 kDa for heavy and light chains, respectively (Figure 1A). Plate-bound Fab A4 is recognized by anti-NY-ESO-1 but not controls such as antibodies to vascular endothelial growth factor (VEGF) or CD20, when analyzed by ELISA (Figure 1B).

Anti-idiotypic Fab A4 binds to cell surface-expressed anti-NY-ESO-1 CAR. Figure 2A shows the schematic representation of expressed CARs. To test whether A4 would also recognize anti-NY-ESO-1 CAR when it is expressed on the surface of the cells, we transfected 293T cells with anti-NY-ESO-1 CAR or anti-CEA CAR constructs, respectively. The expression of CARs was determined by flow cytometry by staining with an antibody against human IgG for all constructs. Transfection efficiencies ranged from 75 to 90%. As expected, anti-idiotypic Fab A4 and HLA-A*0201/NY-ESO-$1_{157-165}$ tetramers bound specifically to anti-NY-ESO-1 CAR only (Figure 2B).

*Affinity measurement of anti-idiotypic Fab A4 and competition with HLA-A*0201/NY-ESO-$1_{157-165}$ tetramer.* The apparent affinity constant (Kd_{app}) of the anti-idiotypic Fab A4 was measured on anti-NY-ESO-1 CAR-transfected 293T cells by analyzing the binding of serially diluted antibody

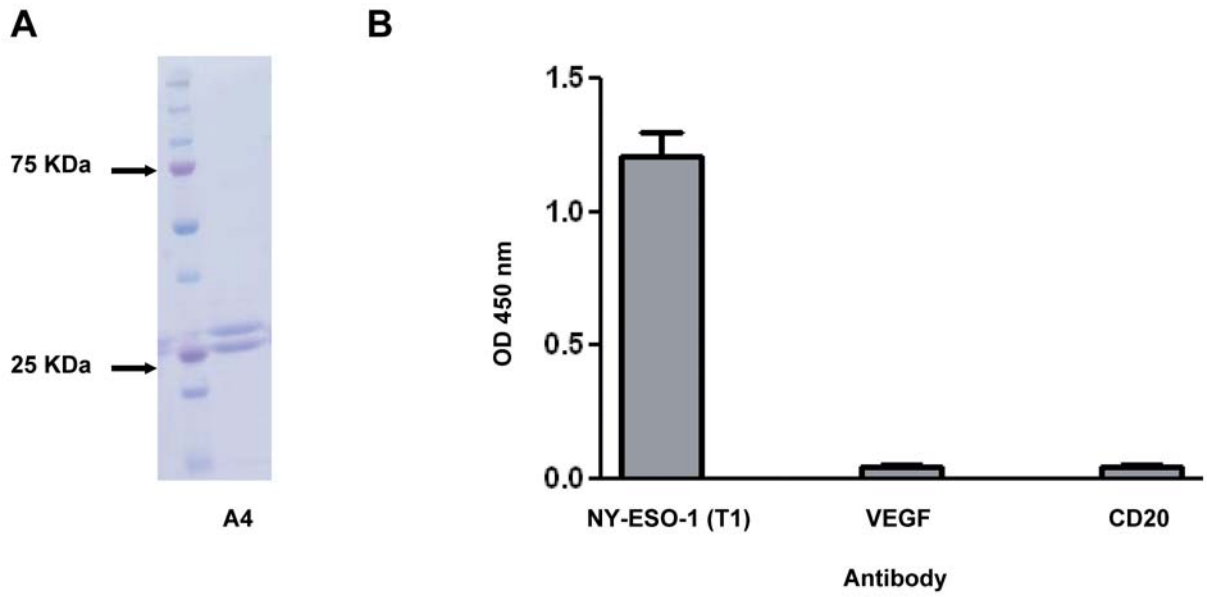

Figure 1. *Expression and purification of soluble anti-idiotypic Fab molecules (A4). A: Purified anti-idiotypic Fab molecules when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A4). B: Binding of soluble purified anti-idiotypic Fab molecules A4 to immobilized anti-HLA-A0201/NY-ESO-1$_{157-165}$ molecules was determined by enzyme-linked immunosorbent assay. Antibodies to B-lymphocyte antigen CD20 (CD20) and vascular endothelial growth factor (VEGF) antibodies were used as control. ***p<0.0001.*

concentrations (Figure 2C). Half-maximum fluorescence intensity was used to calculate the apparent affinity. The calculated binding affinity of the anti-idiotypic Fab A4 to our anti-NY-ESO-1 CAR as shown by the Kdapp values was 200 nM which is comparable to data obtained from surface plasmon resonance (SPR) on a CMS5 chip coated with anti-NY-ESO-1 Fab (data not shown). Next, we analyzed the binding competition of anti-idiotypic Fab molecules with HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer for anti-NY-ESO-1 CAR which was expressed on CD8$^+$ T-cells. HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer binding was inhibited in a dose-dependent manner as shown in Figure 2D. These results indicate that anti-idiotypic Fab A4 competes with HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer for anti-NY-ESO-1 CAR binding and completely blocked tetramer binding to anti-NY-ESO-1 CAR at high Fab A4 antibody concentrations.

Anti-idiotypic Fab A4 activates anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells in vitro. Purified human CD8$^+$ T-cells were transduced by retrovirus-mediated gene transfer with anti-NY-ESO-1 or anti-CEA CAR (21). Anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells specifically secreted IFNγ in the presence of the anti-idiotypic Fab A4, whereas no specific activation was observed for the used control Fab (Figure 3A). The anti-idiotypic induced secretion of IFNγ was specific since only background levels of IFNγ were found when anti-CEA CAR-redirected CD8$^+$ T-cells were stimulated with A4 (Figure 3A).

Next, we compared the activation potential of the anti-idiotypic Fab A4 molecules and recombinant human HLA-A2 molecules pulsed with NY-ESO-1$_{157-165}$ peptide. For that purpose, equimolar concentrations of both molecules were immobilized on cell culture plates and the IFNγ secretion of anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells was determined by ELISA. Anti-idiotypic Fab A4 led to significantly higher levels of IFNγ secretion at non-saturating concentrations (66 nM and 22 nM) when compared with human HLA-A2 molecules pulsed with the NY-ESO-1$_{157-165}$ peptide (Figure 3B).

Anti-idiotypic Fab-dependent expansion of anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells. CD8$^+$ T-cells were incubated with immobilized anti-idiotypic A4 Fab molecules to monitor the effect of these molecules on receptor-triggered proliferation. We compared the antigen-specific expansion of anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells produced by irradiated T2-1B (50 Gy) cells with anti-idiotypic Fab molecules. Changes in CAR-positive T-cells and cell numbers were monitored over a 28-day period. Stimulation with immobilized anti-idiotypic Fab A4 or T2-1B cells induced proliferation of functional anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells (Figure 4B). Both expansion protocols resulted in 96% CAR-positive cells (Figure 4A). There was a more rapid expansion of anti-NY-ESO-1 CAR-redirected CD8$^+$ T-cells in response to anti-idiotypic Fab A4 Fab when compared to irradiated T2-1B cell stimulation (Figure 4C).

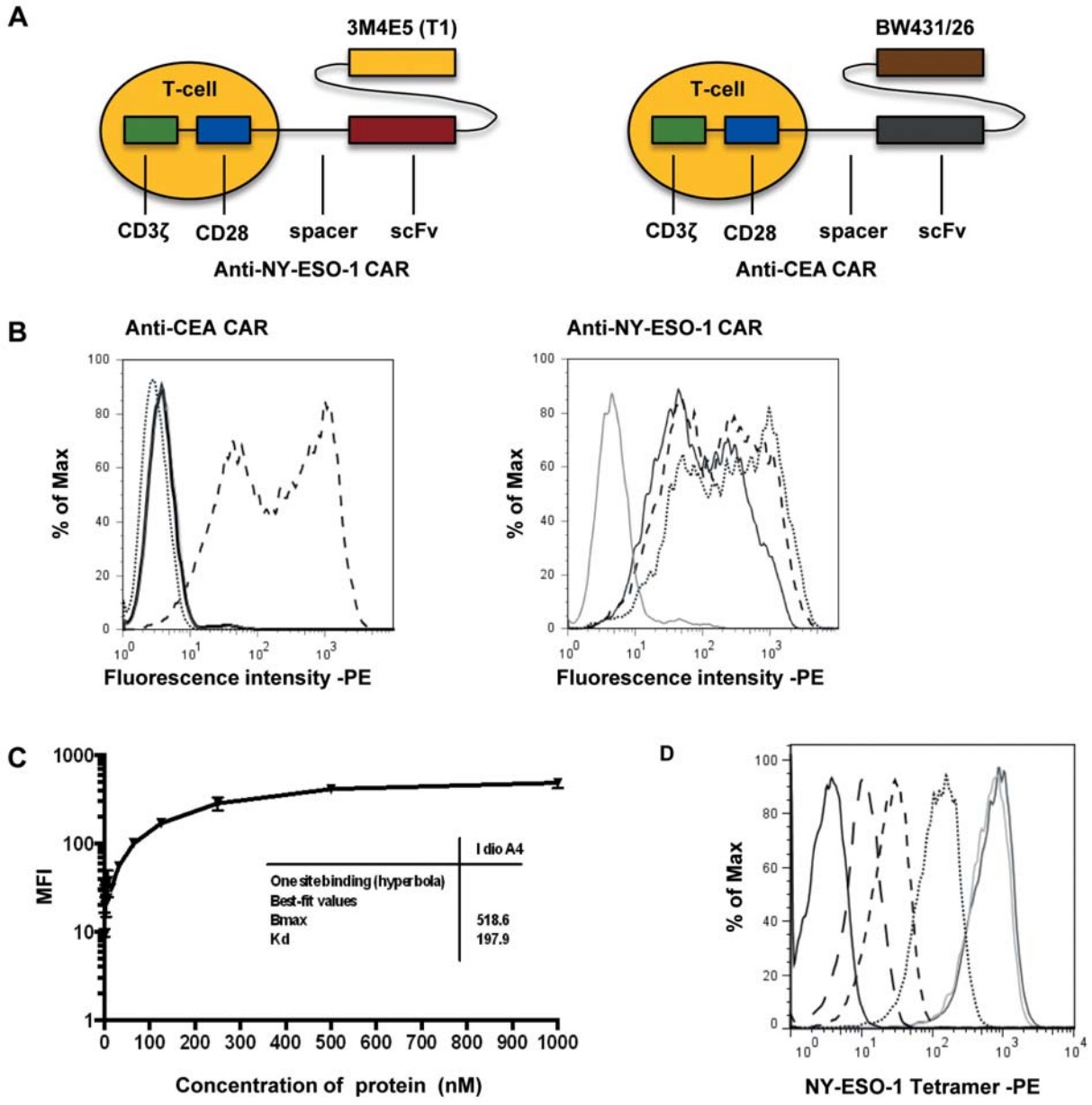

Figure 2. *Binding of anti-idiotypic Fabs to anti-NY-ESO-1 CAR-redirected cells. A: Schematic presentation of redirected T-cells recognizing NY-ESO-1$_{157-165}$ peptide with a cluster of differentiation 28 (CD28) co-stimulatory moiety (anti-NY-ESO-1-CAR) and redirected T-cells recognizing carcinoembryonic antigen (CEA) with a CD28 co-stimulatory domain (anti-CEA-CAR). Single chain fragment variable (ScFv) is the antigen binding region and two antibody constant regions (constant heavy chain CH2 and CH3) represent the immunoglobulin spacer. B: Surface binding of soluble purified Fabs to anti-NY-ESO-1 CAR-transfected 293T cells. 293T cells were transfected with anti-CEA CAR and anti-NY-ESO-1 CAR. Expression of both CARs was determined by staining with antibody to human IgG binding the CH2/CH3 immunoglobulin spacer (dashed line). Correct folding of anti-NY-ESO-1 CAR was determined by staining with HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer- phycoerythrin (PE) (dotted line). His-tagged Fab (A4) binding to anti-NY-ESO-1 CAR was determined by anti-HIS-tag PE-labeled monoclonal antibody (black line). As negative controls, transfected 293T cells stained with an irrelevant His tagged control Fab molecule (light grey). C: For affinity studies, anti-idiotypic Fab molecules were incubated at different concentrations with NY-ESO-1 CAR-transfected 293T cells. Detection was carried out with Penta-His, anti-mouse IgG (H+L) biotin, and streptavidin PE-conjugated antibodies. The half-maximal binding concentration (Kd) was calculated to determine the apparent affinity of anti-idiotypic Fab to anti-NY-ESO-1 Fab. D: Competition between anti-NY-ESO-1 anti-idiotypic Fab molecules and HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer was analyzed. Competition assay was performed with anti-NY-ESO-1 CAR-redirected CD8+ T-cells. Redirected T-cells were saturated with different concentrations of anti-idiotypic Fab A4 (long dash: 1 μg/μl, dash: 0.25 μg/μl, spotted: 0.03 μg/μl) and control Fab molecules (light grey: 1 μg/μl). After 15 min of incubation, HLA-A*0201/NY-ESO-1$_{157-165}$ tetramer was added to the cells. As a positive control, tetramer alone was used without pre-incubation (0.002 μg/μl, dark grey) and an isotypic antibody served as negative control (black: isotype control). Competition was measured by flow cytometry, as described in the materials and methods.*

Phenotypical and functional analysis of CAR-redirected CD8+ T-cells during in vitro expansion. For the phenotypic analysis of expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells, we used a flow cytometric panel to distinguish different populations of central memory T-cells (CCR7+, CD62L+) and effector T-cells (CCR7−, CD62L−) to compare the effect of anti-idiotypic Fab and T2-1B cells on phenotypic change of anti-NY-ESO-1 CAR-redirected CD8+ T-cells (25). The analysis was carried out with redirected T-cells expanded *in vitro* for 28 days. We did not observe significant phenotypic differences in cells between the two stimulation procedures used (Figure 5). As expected, after expansion, the majority of the cells displayed an effector phenotype.

Next, we analyzed the functional capacity of these *in vitro* expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells in response to antigen-specific stimulation. To address this question, we collected anti-NY-ESO-1 CAR-redirected CD8+ T-cells at different time points during the expansion phase and co-incubated them with T2-1B (antigen-specific) and T2-1C (control) cells for 4 h. Expanded anti-NY-ESO-1 CAR-redirected T-cells were fully functional since they secreted cytokines (IFNγ, TNFα and IL-2) in an antigen-specific manner at day 28 (Figure 6). Over time, similar levels of antigen-specific cytokine secretion were observed in both anti-idiotypic and T2-1B expanded redirected T-cells in response to antigen (Figure 7A and B). To prove the cytolytic potential, expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells were co-incubated with T2-1B and T2-1C cells for 24 h. Most importantly, expanded redirected T-cells specifically lysed T2-1B target cells regardless of the expansion protocol used (Figure 8A and B). Considering the number of antigen-specific T-cells required for adoptive T-cell therapy, anti-idiotypic-dependent expansion increased the T-cell number within 28 days by 300-fold. A4 Fab increased the redirected number of T-cell two fold compared to T2-1B expansion.

Antitumor effect of expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells in vivo. Redirected and expanded CD8+ effector T-cells were assessed in a Winn assay to finally demonstrate functionality of antigen-specifically expanded redirected T-cells (26). It has been shown that freshly transduced anti-NY-ESO-1 CAR-redirected CD8+ T-cells exhibited an anti-tumor effect in a protective mouse model (21). In the assay performed here, NSG mice were subcutaneously injected with U266 cells (10×10^6). Subcutaneous co-injection with either 10×10^6 anti-idiotypic (A4) or cell-based (T2-1B) expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells was performed. Tumor growth was measured by the volume of the subcutaneous tumors and by human IgE levels in the mouse serum since the multiple myeloma U266 cell line secretes human IgE, which can be used as a surrogate parameter for cell growth. The control group injected with U266 cells alone started secreting human IgE one week after injection, whereas no IgE secretion was observed in groups treated with anti-idiotypic or and T2-1B-expanded CD8+ T-cells. An increase in IgE levels was observed in control-group mice up to day 35, whereas no IgE secretion was observed in mice injected with anti-NY-ESO-1 CAR-

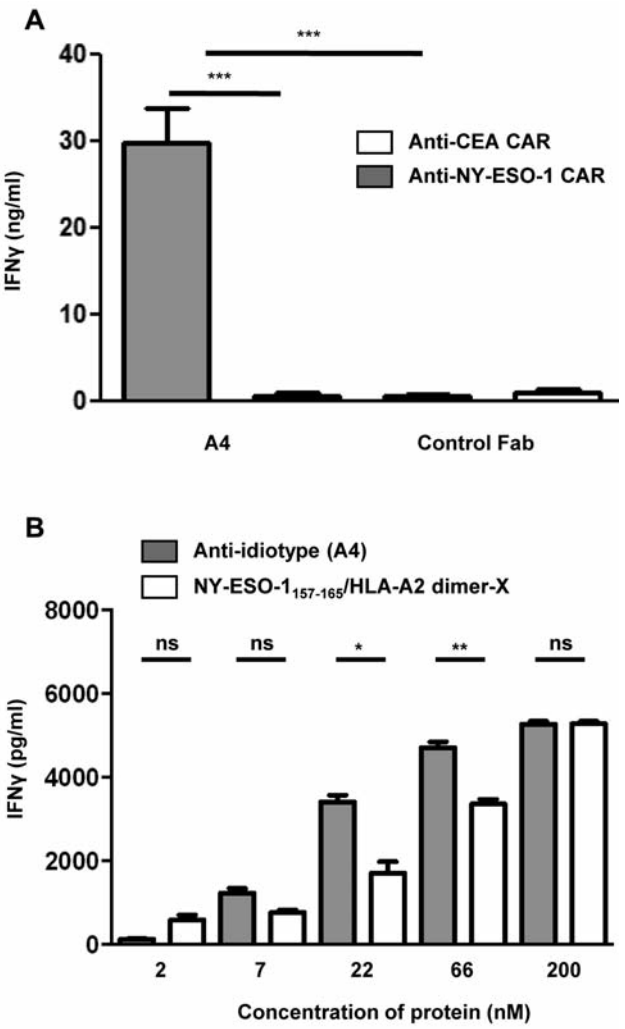

Figure 3. *Anti-idiotypic Fab-dependent activation of anti-NY-ESO-1 CAR redirected cluster of differentiation 8 (CD8+) T-cells. A: Human CD8+ T-cells were retrovirally transduced with anti-NY-ESO-1 CARs and anti-carcinoembryonic antigen (CEA)-CARs. Equal numbers of CAR-positive redirected CD8+ T-cells were cultured in plates coated with anti-idiotype and control Fabs for 24 h. As activation marker, interferon gamma (IFNγ) was measured by enzyme-linked immunosorbent assay (ELISA) in the cell culture supernatant. B: Equal numbers of recombinant HLA-A2 dimer molecules (pulsed with NY-ESO-1157-165 peptide) and anti-idiotypic Fab molecules at different concentrations were coated on cell culture plates. Anti-NY-ESO-1 CAR redirected CD8+ T-cells were added to the plate and after 24 h the cell culture supernatant was collected and secreted IFNγ was measured by ELISA. ***p<0.001, **p<0.01, *p<0.05; not significant (ns).*

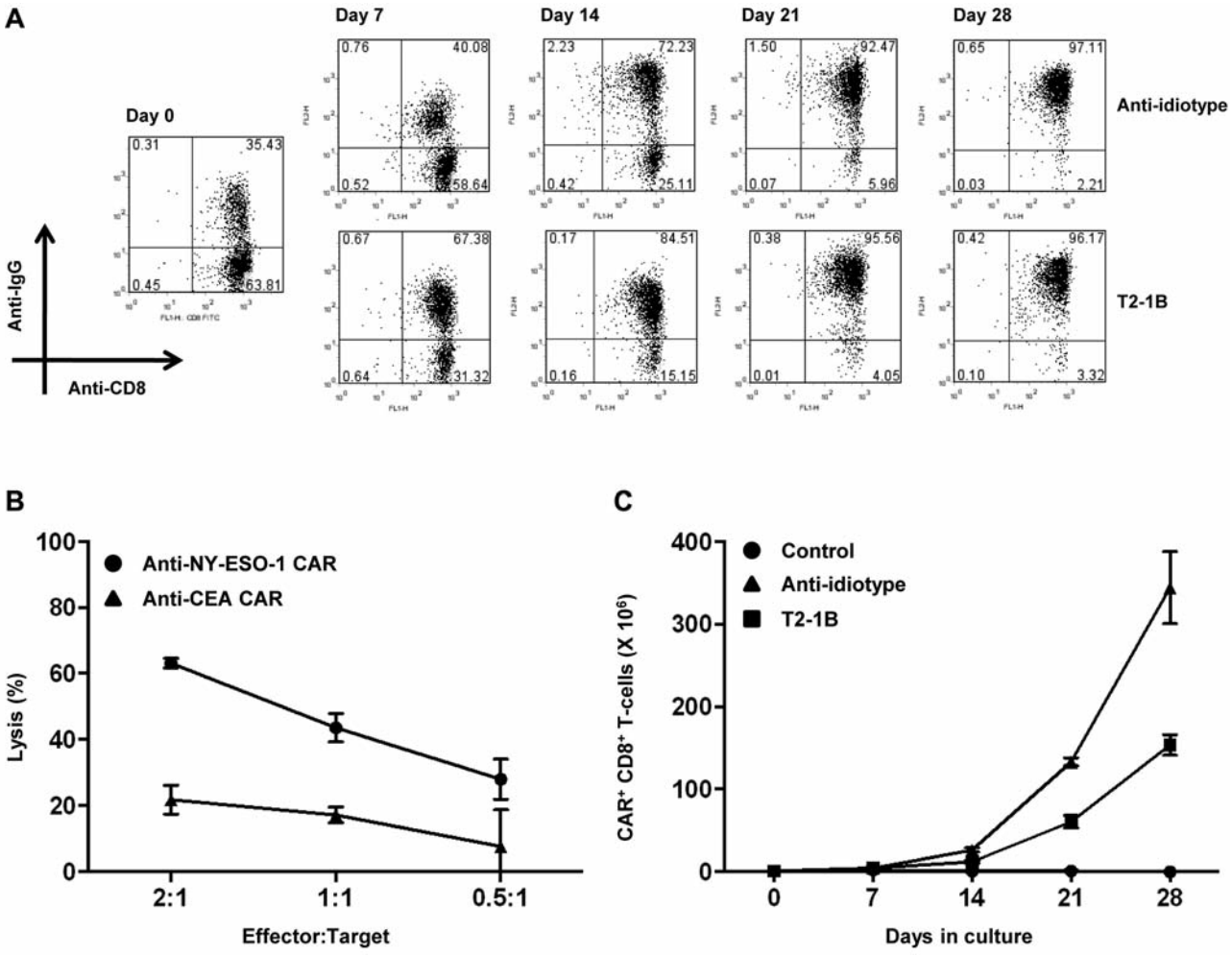

Figure 4. *Antigen-dependent expansion of anti-NY-ESO-1 CAR-redirected CD8+ T-cells. A: At day 0, equal numbers of redirected anti-NY-ESO-1 CAR CD8+ T-cells were co-cultured with HLA-A*0201/NY-ESO-1$_{157-165}$ peptide-expressing T2 cells (T2-1B) at a ratio of 4:1 or with anti-idiotypic Fab A4 (2 μg/ml)-coated flasks in the presence of recombinant human IL-2 (50 IU/ml). To demonstrate antigen-specific functionality before expansion, redirected T-cells were co-cultivated with the endogenously HLA-A*0201/NY-ESO-1$_{157-165}$–positive cell line (21). B: Lysis of U266 cells by anti-NY-ESO-1- or anti-CEA-redirected T-cells was determined at different effector-to-target ratios. C: Receptor positivity and cell numbers were monitored for a period of four weeks. The number of receptor-positive T-cells was analyzed by flow cytometry using phycoerythrin (PE)-conjugated monoclonal antibody to human IgG1 and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to CD8. The number of receptor-positive cells was counted on a weekly basis.*

redirected CD8+ T-cells (Figure 8C). The growth of the subcutaneous tumor clearly indicated the antitumor activity of the expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells. However, we did not observe any differences between anti-idiotypic and T2-1B expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells regarding antitumor activity (Figure 8D).

Discussion

The adoptive transfer of redirected T-cells has recently gained major attention and is being tested in an increased number of patient cohorts, mainly because of its clinical success in patients with advanced-stage cancer (9, 10). The procedure offers much promise to the field of tumor immunotherapy but still requires optimization and selective expansion of tumor antigen-specific T-cells at a GMP level.

It has been shown that sufficient numbers of T-cells can be generated for adoptive T-cell therapy either by TILs or redirected T-cells *in vitro* (27). However, being cell-based expansion techniques, both these approaches often suffer from lack of selective expansion of antigen-specific T-cells and lead to the activation of non-specific T-cells due to polyclonal stimulation (28).

Therefore, to mitigate these limitations, we herein describe to our knowledge for the first time, an anti-idiotypic antibody-dependent expansion of peptide-specific

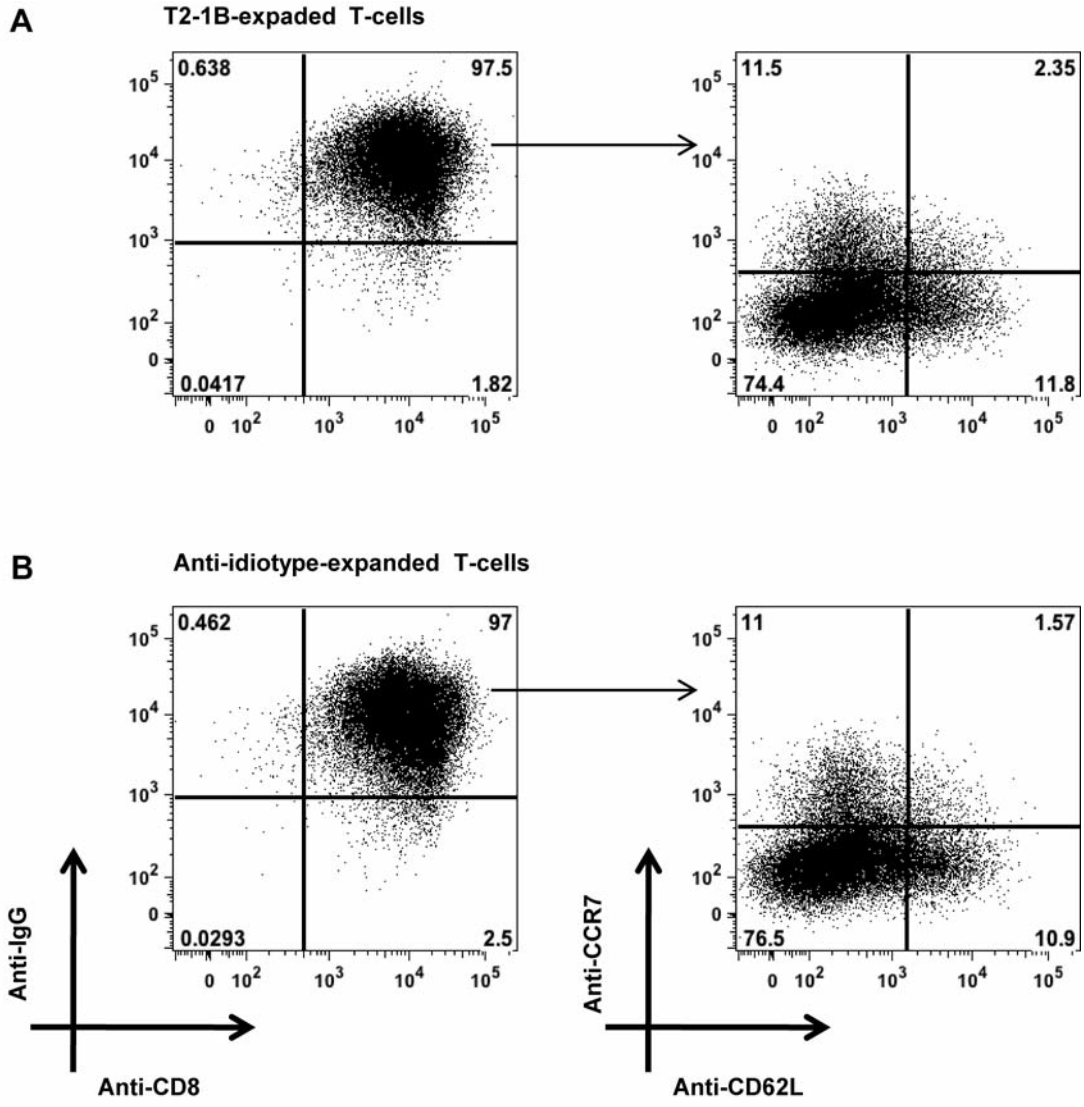

Figure 5. *Phenotypical characterization of expanded redirected T-cells. Redirected T-cells were identified by expression of CD8 and immunoglobulin spacer. Memory and effector phenotype were displayed by cluster of differentiation 62L (CD62L) and C-C chemokine receptor type 7 (CCR7) expression on anti-idiotypic- and T2-1B expanded anti-NY-ESO-1 CAR redirected CD8+ T-cells.*

NY-ESO-1 CAR (CAR derived from TCR-like antibodies). The anti-idiotypic expansion method has several advantages when compared with the other expansion protocols mentioned. Primarily, it mimics the antigen recognized by NY-ESO-1 CAR and leads to a selective expansion of antigen-specific T-cells, unlike polyclonal expansion using rapid expansion protocols (REP) or anti-CD3/CD28 beads (19, 29). Secondarily, since production of antibodies of GMP grade is a well-established technique, the production of anti-idiotypic antibody molecules at sufficient numbers of GMP grade for clinical use can be established based on common knowledge.

In our present study, anti-idiotypic antibody Fab A4 was selected from a phage display library because of its specific binding capacity towards anti-HLA-A*0201/NY-ESO-$1_{157-165}$ antibody 3M4E5 (22) and a 3M4E5 Fab variant (T1) that had been further improved by rational design to increase affinity for the HLA-A*0201/NY-ESO-$1_{157-165}$ complex. Mutation of two amino acids in the variable light chain sequence of the 3M4E5 Fab led to a 20-fold increased affinity (20). Due to the higher affinity, we used the T1 scFv variant to redirect T- cells by a CAR construct and have recently demonstrated its functional activity *in vitro* and *in vivo* (21). The anti-idiotypic antibody A4 and the HLA-

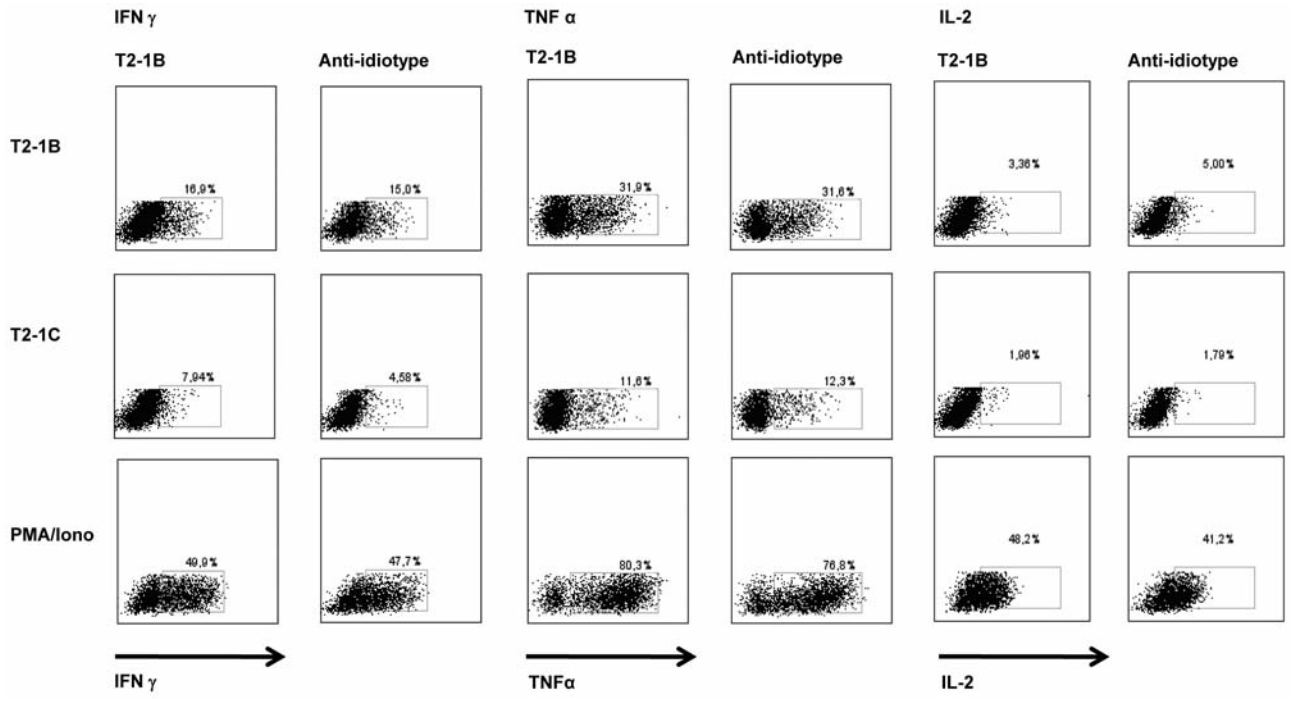

Figure 6. *Comparison of the percentage of functional antigen specific CAR-redirected CD8+ T-cells after 28 days of expansion with T2-1B cells or anti-idiotypic Fab A4. Antigen-specific cytokine release was determined by intracellular staining. T2-1B and anti-idiotypic Fab A4-expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells were incubated with T2-1B (antigen-specific) and T2-1C (control) cells for 4 h. Cells were stimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycine as positive control. The percentage of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2)-positive cells of T2-1B-cell- and anti-idiotypic Fab A4-expanded CAR-positive CD8+ T-cells was measured.*

A*0201/NY-ESO-$1_{157-165}$ tetramer bound to the anti-NY-ESO-1 CAR in a comparable manner. This indicated the antigen-mimicking effect (HLA-peptide complex) of the anti-idiotypic antibody (30, 31). Furthermore, Fab A4 and the HLA-A*0201/NY-ESO-$1_{157-165}$ tetramer competed for binding to the anti-NY-ESO-1 CAR. Taken together, these data clearly indicate the property of the A4 anti-idiotypic antibody to mimic the antigen recognized by T1 and to selectively expand antigen-specific CAR-expressing T-cells.

Comparison of Fab A4 anti-idiotypic antibody with transfected T2 cells (T2-1B) known to express large amounts of NY-ESO-$1_{157-165}$ peptide (as antigen-presenting cells) resulted in a more than 300-fold increase of antigen-specific NY-ESO-1 CAR-redirected $CD8^+$ T-cells after Fab A4 stimulation, which is double the number of T2-1B expanded T- cells and comparable to other expansion protocols used in preclinical or clinical development (32). However, currently, no anti-idotypic expansion protocol is available for CARs derived from TCR-like antibodies.

Adoptive transfer of T-cells in patients requires approximately 10^8-10^{11} T-cells/m^2 (33, 34). Currently available protocols for the *in vitro* expansion of T-cells use REP or anti-CD3/CD28 bead stimulation to successfully generate sufficient numbers of tumor-specific T-cells (29, 35). It has been shown that peptide stimulation prior to the anti-CD3/CD28 bead stimulation further increases the number of antigen-specific T-cells (36). Even though increased numbers of antigen-specific T-cells can be generated by loading peptides onto artificial or natural antigen-presenting cells by antigen-specific clonal expansion in the presence of anti-CD28 co-stimulatory molecules (37), it is difficult to produce antigen-specific T-cells using these methods under GMP conditions. When anti-CD3/CD28 bead stimulation was used to expand cytomegalovirus (CMV), specific T-cells *ex vivo*, this approach resulted in low frequencies of CMVpp65 peptide-specific T-cells, underlining the non-specific nature of anti-CD3/CD28-based activation (38). In contrast, our anti-idiotypic antibody approach fulfills the most required benchmark for efficient T-cell therapy: antigen-specific clonal expansion of functional T-cells.

Adoptive transfer of tumor-specific memory T-cells has been shown to be superior in regard to tumor protection. Antigen-specific T-cells expanded *in vitro* with artificial antigen-presenting cells comprise of central memory ($CCR7^+$ $CD62L^+$) and effector populations ($CCR7^-$ $CD62L^-$).

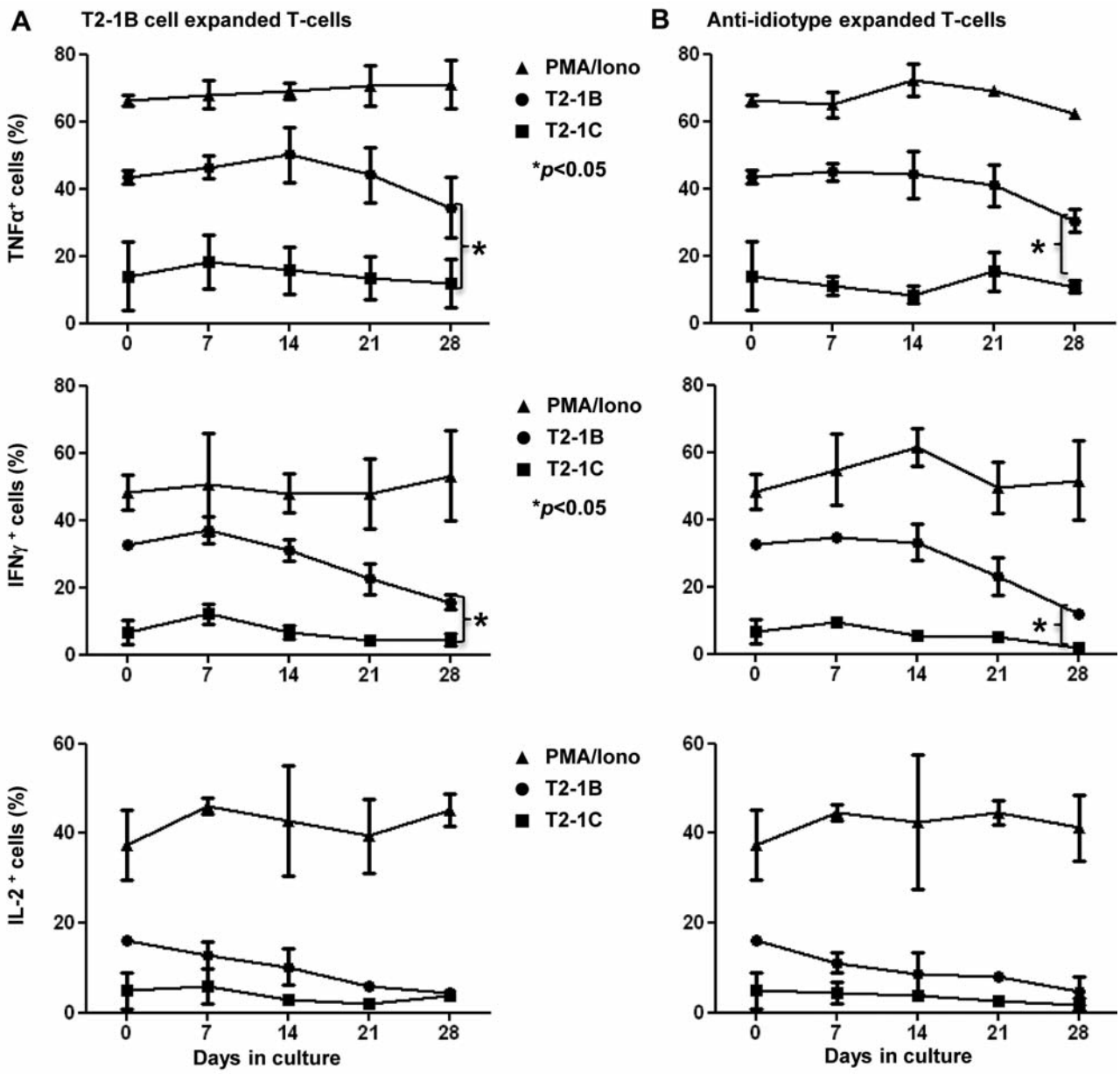

Figure 7. *Activation of expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells. The percentage of functional antigen-specific CAR-redirected CD8+ T-cells was determined by intracellular cytokine staining for a period of 28 days. T2-1B- and anti-idiotypic Fab A4- expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells were incubated with T2-1B (antigen-specific cells) and T2-1C (control cells) cells for 4 h. As positive control, cells were stimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycine. The percentage of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2)-positive cells of T2-1B cell (A) and anti-idiotypic Fab A4 (B) expanded CAR-positive CD8+ T-cells was measured at different time points of T-cell culture. *p<0.05.*

Adoptive transfer of these *in vitro* expanded T-cells showed establishment of an anti-tumoral immunological memory (39). The expansion of anti-NY-ESO-1 CAR-redirected $CD8^+$ T-cells with anti-idiotypic Fab antibody resulted predominantly in effector T-cells ($CCR7^-$ $CD62L^-$). For the clinical testing of safety, the use of terminally differentiated effector T-cells can be of advantage since the expected anti-tumor effect as well as potential side-effects, would be transient due to the natural fate of these cells. Nevertheless, we observed clear antigen-specific functionality of the expanded T-cells. Redirected T-cells demonstrated cytotoxic activity over a 28-day expansion period. However, antigen-specific cytokine secretion started to decrease after 14 days of expansion. This effect was seen for anti-idiotypic and T2-1B expanded T-cells.

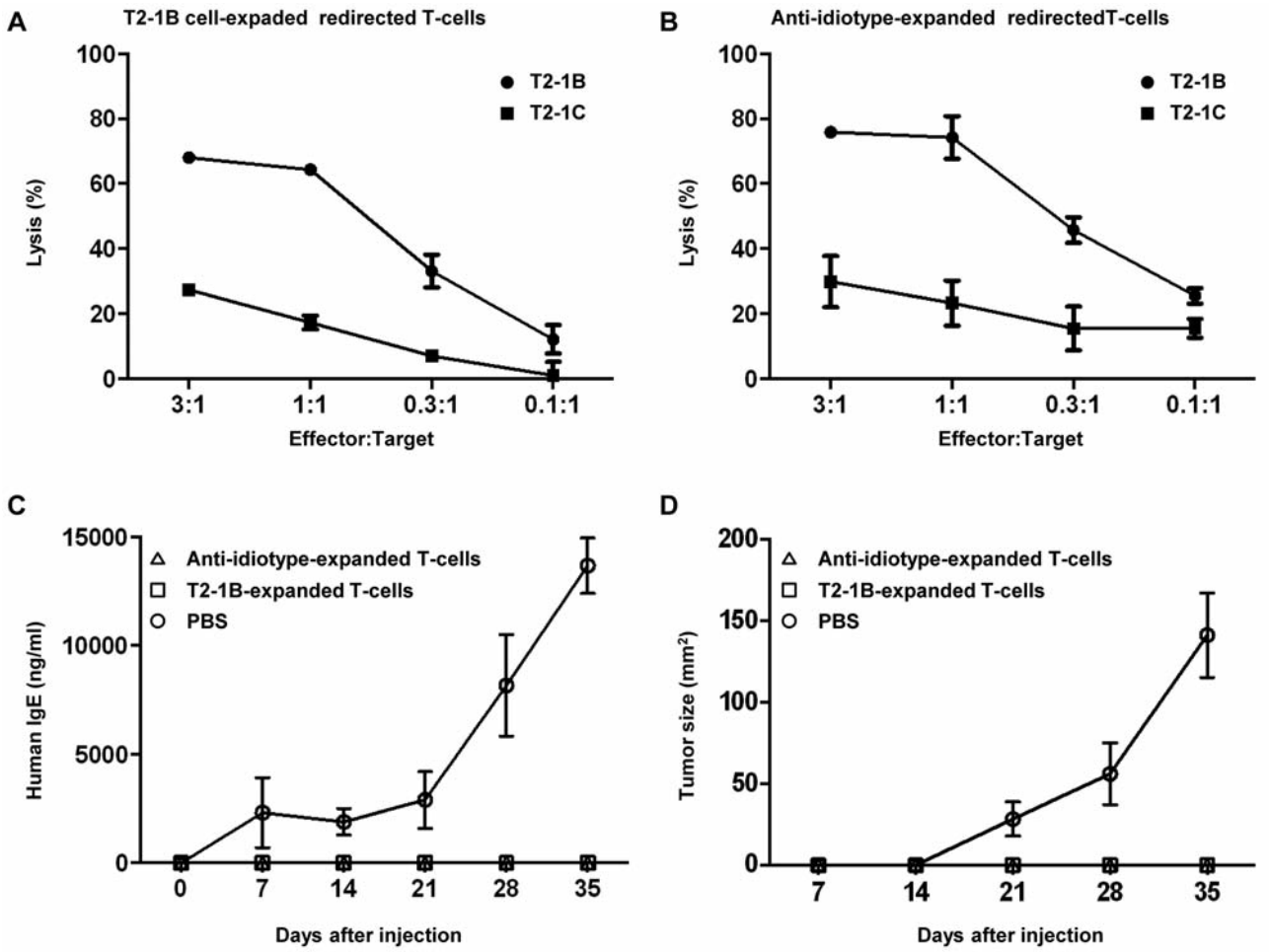

Figure 8. *The cytotoxic activity of expanded redirected T-cells was assessed by measuring the viability of target cells by a tetrazolium salt-based (XTT) assay after 24 h. T2-1B- (A) and anti-idiotypic Fab A4-expanded (B) anti-NY-ESO-1 CAR redirected CD8+ T-cells were incubated with T2-1B (antigen-specific cells, HLA-A*0201/NY-ESO-$1_{157-165}$) or T2-1C (control cells, HLA-A*0201/NY-ESO-$1_{155–163}$) at different effector-to-target cell ratios. Mice were subcutaneously injected with 10^7 HLA-A*0201/NY-ESO-$1_{157-165}$–positive (21) U266 cells and 10^7 anti-idiotype- or T2-1B-expanded anti-NY-ESO-1 CAR-redirected CD8+ T-cells. A group treated with phosphate buffered saline (PBS) served as control. Tumor growth was monitored by measuring serum IgE levels (C) and tumor size (D) (n=5 per experimental group).*

We further investigated the effect of our anti-idiotypic antibody expanded T-cells *in vivo* by using the natural NY-ESO-1 antigen-expressing multiple myeloma cell line U266. Our previous study had demonstrated an anti-tumor effect of anti-NY-ESO-1 CAR *in vivo* (21). Functionality of the anti-idiotypic Fab antibody-expanded T-cells *in vivo* was confirmed using a subcutaneous U266 tumor model. Growth of U266 cells in NSG mice was monitored by measuring serum human IgE levels as a surrogate marker (40). Serum IgE levels were detected in control mice from day 7 after U266 injection but were not detectable in mice treated with expanded CD8+ T-cells even after 35 days. Furthermore, we observed a direct correlation between tumor growth and secreted human IgE levels in control mice, validating phenotypic tumor growth with functional surrogate marker increase. Multiple myeloma is a valid model for the analysis of NY-ESO-1-directed immunotherapeutic approaches since the NY-ESO-1 protein is frequently expressed in myeloma cells of patients with advanced-stage disease or cytogenetic abnormalities (41). In addition, NY-ESO-1 protein-positive patients mount a spontaneous NY-ESO-1 peptide-specific humoral and cellular immune response, supporting NY-ESO-1 as a valid target antigen for this disease. A recently presented study using redirected autologous T-cells expressing a high affinity TCR specific for NY-ESO-1 administered to patients with advanced myeloma demonstrated an overall response rate greater than 80%, with mild toxicity (42). TCR-transduced cells were detected

at 1% in the peripheral blood and bone marrow for up to one year after transfusion, demonstrating long-term persistence.

Taken together, we conclude that our cell-based and cell-free expansion protocols for redirected T-cells resulted in equally functional T-cells. However, the cell-free anti-idiotype-based approach resulted in significantly higher numbers of antigen-specific T-cells. The higher cell yield and the cell-free process are strong arguments to establish this protocol in a GMP process in preparation for clinical trials testing the HLA-A*0201/NY-ESO-$1_{157-165}$ complex-specific redirected T-cells for the experimental treatment of patients with HLA-A*0201-positive NY-ESO-1-expressing multiple myeloma. Hence, the anti-idiotypic antibody approach might offer one step further along the path for more effective personalized medicine and might have implications in the future development of adoptive T-cell therapy.

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