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CHAPTER 6

The novel bispecific diabody αCD40/αCD28 strengthens leukemic dendritic cell induced T cell reactivity

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Abstract

Dendritic cell (DC)-based immunotherapy faces new challenges since efficacy of DC vaccines in clinical trials has been inconsistent. Strategies to improve immune responses induced by DC are currently being explored. We have recently shown the feasibility of generating fully functional DC from acute myeloid leukemia (AML) blasts, but with varying expression levels of the important co-stimulatory molecule CD86. To overcome this variability, we developed a novel bispecific diabody simultaneously and agonistically targeting CD40 on AML-DC and CD28 on *naïve* T cells. Beside optimization of CD28-mediated signaling, the resulting cellular cross-linking was also hypothesized to increase the strength and duration of T cell/AML-DC interactions, thus increasing T cell responsiveness to AML antigens. Here we report that the αCD40/αCD28-bispecific diabody indeed displays binding to its target antigens and provokes increased T cell–DC cluster formation. The αCD40/αCD28 diabody is capable of increasing T cell proliferation induced by AML-DC as well as the induction of DC maturation. Importantly, priming efficacy of tumor-specific cytotoxic T cells can also be improved by cross-linking AML-DC and T cells with the αCD40/αCD28 diabody. We propose that the αCD40/αCD28-bispecific diabody can serve as a potent therapeutic tool to effectively augment anti-tumor T cell responses elicited by AML-DC.

Introduction

Dendritic cell (DC)-based immunotherapy offers a promising approach to elicit specific T cell responses directed against tumor-specific or tumor-associated antigens. Therapeutic and protective anti-tumor immunity induced by DC presenting tumor-specific antigens, have been observed in various mouse models. Despite an ever increasing number of clinical DC-based tumor vaccination trials, the therapeutic efficacy of this novel approach has recently been questioned. Clinical responses seem limited with anti-tumor responses observed in approximately half of the trials (1,2). Therefore, current research tends to focus on strategies that improve the efficacy of DC vaccines.

We have recently shown the feasibility of generating DC from Acute Myeloid Leukemic (AML) blasts (3,4). Indeed, AML-DC maintain their intrinsic leukemic characteristics and obtain full antigen presenting capacity (3-7). AML-DC exhibit all the functions mandatory to elicit an immune response *in vivo*, such as migration to lymph node–associated chemokines, induction of T cell proliferation and, most importantly, induction of leukemia-specific cytotoxicity (7-10). Nevertheless, the heterogeneity of this disease type was reflected in variable levels of the key co-stimulatory molecule CD86 on the surface of the resulting AML-DC. We therefore set out to overcome this variability in CD86 levels through the use of bispecific antibody constructs.

Bispecific antibodies consisting of binding sites for two different antigens are used to cross-link two different antigen-bearing cells (11,12). By bridging two cell types they are capable of redirecting an effector system, such as T cells, towards tumor cells or DC, thus facilitating signaling between these cells that could further improve effector T cell functions. Bispecific antibodies have mainly been produced using murine hybridomas or by chemical cross-linking. However, clinical trials are hampered by human anti-murine antibody responses and release of inflammatory cytokines (13-15). Alternatively, bispecific diabodies consisting of two single chain variable fragments (scFv) could be

used. These bispecific diabodies are formed by non-covalent association of 2 single chain-fusion products of variable light (V_L) and variable heavy chains (V_H) of different specificity (16). This product is potentially less immunogenic and can easily be produced in bacteria in large quantities (17,18). Simultaneous targeting of tumor cells and lymphocyte populations has been the main topic for bispecific antibody usage (19). We were interested in facilitating the binding between T cells and AML-DC, potentially resulting in increased signaling between these cell types and improved T cell responses induced by the AML-DC. Since CD40 was amply expressed on AML-DC, we opted to agonistically target this marker, while simultaneously facilitating agonistic binding to CD28 on naïve T cells, through the use of a diabody consisting of both an anti-CD40 scFv and an anti-CD28 scFv. This strategy allows for the compensation of variable CD86 expression levels on the AML-DC surface while ensuring efficient CD40-mediated maturation of the AML-DC. Indeed, here we show that the newly developed αCD40/αCD28 diabody significantly improves T cell proliferation and priming efficacy of tumor-specific cytotoxic T cells, while simultaneously inducing maturation of AML-DC.

Materials and methods

Construction of αCD40/αCD28 bispecific diabody

The G28-5 anti-human CD40 monoclonal antibody as well as cloning of the single chain variable fragments (scFv) of G28-5 into the bacterial expression vector pAB1, have been described previously (20-22). The pHOG_28-19 and pHOG_19-28 expression vectors encode V_H and V_L of 15E8, anti-human CD28, respectively, and were provided by Dr. S. Kipriyanov (Affimed, Heidelberg, Germany). Amplification by polymerase chain reaction (PCR) of the V_L and V_H domains from scFv G28-5 was performed by using the following primers: V_L sense primer: 5'-ACACCCAAGCTTGGCGGTGATGCTGTGATGACCCAAAATCC, V_L antisense primer: 5'-GATCCAGCGGCCGCAGCATCAGCCCGTTTTATTTCCAGCGTGG, V_H sense primer: 5'-AGCCGGCCATGGCGGATATACAGCTTCAGCAGTC, V_H antisense primer: 5'-ACCGCCAAGCTTGGGTGTTGTTTTGGCTGAGGAGACGGTGACTGAGG. PCR fragments of 381 base pairs (bp) and 377 bp, respectively, were isolated, using a gel extraction kit (Qiagen, Venlo, the Netherlands). The V_L amplimer contained a *Hind*III restriction endonuclease site in the sense primer and a *Not*I site in the antisense primer (underlined) which were used to digest the fragment. The V_H amplimer contained a *Nco*I site in the sense primer and a *Hind*III site in the antisense primer (underlined) and were used to digest the G28-5 V_H fragment. The digested αCD40 V_L fragment was isolated and ligated into pHOG_28-19 linearized with *Hind*III and *Not*I, resulting in pHOG-V_H^{CD28}-V_L^{CD40}. Subsequently, the αCD40 V_H fragment was isolated and ligated into pHOG_19-28, linearized with *Nco*I and *Hind*III, resulting in pHOG-V_H^{CD40}-V_L^{CD28}. After digestion of pHOG-V_H^{CD40}-V_L^{CD28} with *Bgl*II and *Xba*I, a fragment of 873 bp, encoding the hybrid scFv V_H^{CD40}-V_L^{CD28} was isolated and ligated to *Bgl*II and *Xba*I linearized plasmid pHOG-V_H^{CD28}-V_L^{CD40}, resulting in the final expression vector pKID_40x28.

Bacterial expression and purification of the αCD40/αCD28- bispecific diabody

The bispecific diabody was expressed and purified as previously described (23). In short, a single colony of E. Coli XL1-Blue (Stratagene, La Jolla, CA) transformed with pKID_40x28 was cultured overnight in YT medium containing 100 μg/ml ampicillin and 2% glucose (YT_{GA}) at 37°C. The culture was diluted 1:40 with fresh YT_{GA} and cultured at 37°C to an optical density (OD) 600nm of 0.8-0.9. Bacteria were pelleted by centrifugation at 1500g for 10 minutes at 20°C and resuspended in an equal volume of fresh YT medium supplemented with 100 μg/ml ampicillin and 0.4 M sucrose. Expression was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.2 mM. The culture was continued at 20°C for 16 hours. Cells were harvested by centrifugation at 6200g for 20 minutes at 4°C. Bacterial pellet was resuspended, in 5% of the original volume, in ice-cold 50 mM Tris-HCl, 20% (w/v) sucrose, 1 mM EDTA (pH 8.0). After incubation for 1 hour on ice, with occasional stirring, cells were centrifuged at 30.000g for 40 minutes at 4°C. Supernatant was collected and thoroughly dialyzed against 50 mM Tris-HCl, 20% (w/v) sucrose, 1 mM EDTA (pH 7.0), using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL). The bispecific diabody was purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) beads according to protocols of the manufacturer (Qiagen). Subsequent to purification, the product was extensively dialyzed against 50 mM NaH_2PO_4, 0.5 M NaCl (pH 8.0). Analysis of the product was done by reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent Coomassie Brilliant Blue staining or Western Blot on a nitrocellulose membrane according to standard protocols. Staining was performed using anti-His_6 monoclonal antibody conjugated to horseradish peroxidase (Roche, Woerden, the Netherlands). Concentration of the purified proteins were determined by using the Bradford assay (24). To control for confounding traces of endotoxins of the isolated αCD40/αCD28 bispecific diabody, proteins derived from untransformed bacteria were used as controls. The untransformed bacteria underwent the same expression induction and purification procedure as pKID_40x28 transformed bacterial cells. Proteins derived from these bacteria will be further referred to as control protein.

In vitro generation of AML-DC

Blood or bone marrow samples of patients newly diagnosed with AML were obtained after informed consent. AML-DC were generated from fresh or thawed mononuclear cells of AML samples using GM-CSF, TNF-α, SCF, Flt-3L, IL-3 and IL-4 as described previously (3,4). Isolated peripheral blood mononuclear cells derived from healthy donors were used to generate monocyte-derived DC (MoDC). Monocytes were obtained by plastic adherence and subsequently differentiated into DC by 7 days of culture in presence of GM-CSF and IL-4 as previously described (25).

To examine the DC maturation potential of the bispecific diabody, immature AML-DC were incubated with the bispecific diabody (10μg/ml) for an additional 48 hours. As a control, also CD40L (100ng/ml, Alexis Biochemicals, Lausen, Switzerland) in combination with an enhancer (1μg/ml, Alexis Biochemicals) and G28-5 MoAb (10μg/ml) were used to induce maturation. Maturation with a standard mixture of inflammatory cytokines; 50 ng/ml TNF-α, 25 ng/ml IL-1β, 100 ng/ml IL-6 and 1 μg/ml PGE_2 was used as a reference, as described before (8).

Cell number and viability were determined by tryphan blue dye exclusion. The percentage of viable, apoptotic and necrotic cells was evaluated by flowcytometry using 3nM Syto 16 (Molecular Probes, Eugene, OR) and 7-actinomycin (7-AAD, ViaProbe, BD Pharmingen, San Diego, CA) (26).

In vitro generation of MUTZ-3-derived DC

MUTZ-3 DC were generated as described (27). Briefly, DC progenitors derived from the AML cell line MUTZ-3 were cultured in 12 well tissue culture plates at a density of $1x10^5$ cells/ml in MEM-α medium without 5637-conditioned medium in the presence of 100 ng/ml GM-CSF (Schering-Plough, Madison, N.J), 20 ng/ml IL-4 (R&D systems, Abingdon, UK) and 2.5 ng/ml TNF-α (Strathmann Biotec, Hamburg, Germany) for 7 days. Every 3 days new cytokines were added. At day 7, maturation of MUTZ-3 DC was induced by adding a cytokine cocktail consisting of 50 ng/ml TNF-α, 100 ng/ml IL-6, 25 ng/ml IL-1β, and 1 μg/ml PGE_2.

Antibodies, tetramers and flow cytometry

Prior to and subsequent to culture AML blasts, AML-DC and MoDC were characterized by four-color flow cytometric analysis on a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA) using the following monoclonal antibodies (MoAbs): FITC-labeled CD86 (Pharmingen), CD1a (Sanquin, Amsterdam, the Netherlands), CD54 (DAKO, Glostrup, Denmark), PE-labeled CD40 (Immunotech, Marseille, France), CD80, CD83 (Immunotech), PerCP-labeled CD45, anti-HLA-DR, APC-labeled CD34, CD38 and CD14.

T cells were analyzed using FITC-labeled CD28 (DAKO, Glostrup, Denmark) and FITC- and PerCP-labeled MoAbs directed against human CD8β (Immunotech, Marseille, France) and CD8α. PE- and/or APC-labeled HLA-A2 tetramers (Tm) presenting the $hTERT_{988Y}$ epitope were kindly provided by Dr. E. Hooijberg (Dept Pathology, VU University medical center, Amsterdam, The Netherlands) and prepared as described previously (28). Antibody and/or tetramer staining was performed in PBS supplemented with 0.1% BSA and 0.02% natrium-azide for 30 minutes at 4ºC and 15 minutes at 37 ºC respectively. To exclude dead cells in flow cytometric tetramer analysis, 0.5 μg/ml propidium iodide (ICN Biomedicals, Zoetermeer, The Netherlands) was used. Unless stated otherwise MoAbs were purchased from Becton Dickinson.

Aggregation assay

AML-DC were incubated with αCD40/αCD28- bispecific diabody (10μg/ml) for 20 minutes at room temperature. Subsequently, unbound diabody was removed by washing with PBS containing 0.1% human serum albumin (HSA). Labeled AML-DC were cocultured with allogeneic T cells in a 1:5 ratio in round bottom polystyrene tubes (Falcon, Becton Dickinson) and centrifuged for 1 minute at 500 rpm, enabling direct cell contact. After 30 minutes of incubation at 37°C, cells and clusters were analyzed and counted by two independent observers, using a microscope. Clusters were defined as 2 or more T cells connected to 1 AML-DC. Counting was performed in a blinded manner. As controls, prior to incubation with the bispecific diabody AML-DC were incubated with G28-5 MoAb to occupy CD40.

Similarly, T cells were incubated with CD28 MoAb (Sanquin, Amsterdam, the Netherlands) prior to incubation with AML-DC labeled with bispecific diabody.

Mixed lymphocyte reactions

To assess the ability of the bispecific diabody to increase T cell proliferation induced by AML-DC a mixed lymphocyte reactions (MLR) were performed in a 96-well round-bottomed plate (Costar). Peripheral blood mononuclear cells isolated from a buffy-coat from one donor were used as responder cells at a fixed concentration of 5 x 10^4 per well. The use of one PBMC donor was chosen to enable comparison between the MLR of different AML-DC cultures. AML-DC were irradiated at 30 Gy. After coculture of the stimulator cells at different stimulator/responder (S/R) ratios with peripheral blood mononuclear cells for 5 days, each well was pulsed with 0.4 μCi [^{3}H] thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 18 hours. Cocultures were harvested onto a fiberglass filter mat and analyzed for the incorporation of [^{3}H] thymidine in a liquid scintillation counter (Wallac, Turku, Finland).

Primary CTL induction in vitro

Antigen-specific CTL were generated as described (29,30). Briefly, CD8β$^+$ CTL precursors were isolated from PBMC of an HLA-A2$^+$ healthy donor by positive selection using MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany). For this purpose, total PBMC were stained with unlabeled anti-CD8β MoAb (Immunotech) and microbead-conjugated anti-mouse IgG Abs (Miltenyi Biotec). Mature MUTZ-3 DC, prepared as described above, were loaded with 1 μg/ml HLA-A2-restricted peptide hTERT$_{988Y}$ (YLQVNSLQTV, i.e. the P1Y heteroclitic variant of hTERT$_{988}$ (31,32)) in the presence of 3 μg/ml β2-microglobulin (Sigma-Aldrich, St. Louise, MO) for 2-4 hours at room temperature and irradiated (40 Gy). Next, peptide-loaded MUTZ-3 DC were pulsed with either control protein or αCD40/αCD28-bispecific diabody for 30 minutes at 4ºC at a concentration of 28 μg/ml. 1x10^5 peptide- and bispecific diabody- pulsed MUTZ-3 DC were cultured for 10 days with 1x10^6 CD8β$^+$ CTL precursors and 1x10^6 irradiated (80 Gy) CD8β$^-$ PBMC in Yssel's medium (33) supplemented with 1% hAB serum (ICN Biochemicals), 10 ng/ml IL-6 and 10 ng/ml IL-12 in a 24 well tissue-culture plate. At day 1, 10 ng/ml IL-10 (R&D Systems) was added. After 10 days, CTL cultures were re-stimulated with 1x10^5 fresh peptide (10 ng/ml) and bispecific diabody- or control protein-pre-incubated MUTZ-3 DC, in the presence of 5 ng/ml IL-7 (Strathmann Biotec). From day 17, CTL cultures were stimulated weekly with 1x10^5 peptide-loaded JY cells. Two days after each restimulation, 10 U/ml IL-2 (Strathmann Biotec) was added. One day prior to each restimulation, a sample was taken and analyzed by flow cytometry using both PE- and APC-labeled tetramers presenting the relevant epitope. Tetramer-positive CTL were isolated by tetramer-guided MACS and expanded by stimulating weekly with irradiated feeder-mix consisting of allogeneic PBMC and JY cells in Yssel's medium supplemented with 100 ng/ml phytohaemagglutin (PHA; Murex Biotech, Dartford, U.K.) and 20 U/ml IL-2.

CTL avidity analysis

Functional avidity of the generated CTL was determined by a flow cytometric degranulation assay. As a marker for degranulation, the cumulative exposure of granular membrane protein CD107a (also known as lysosomal-associated membrane protein-1 (LAMP-1)) on the cell surface of a responding antigen-specific T cell was measured by flow cytometry. For this purpose, the $hTERT_{988Y}$-specific T cells were stimulated with JY target cells, which were loaded with 10-fold dilutions of the $hTERT_{988Y,}$ for 5 hours at 37°C in a 1:1 ratio in the presence of anti-CD107a-PE (BD Biosciences) and 4 μM monensin (Sigma). Following stimulation, cells were washed, stained with APC-labeled tetramer and FITC-labeled anti-CD8 mAb respectively and analyzed on a FACScalibur.

Statistics

The Student's t-test was used to compare differences between cultures in the presence or absence of the αCD40/αCD28 bispecific diabody. P-values <0.05 were considered significant.

Results

Construction and production of the αCD40/αCD28- bispecific diabody

To target AML-DC and T cells simultaneously, a bispecific diabody was constructed with dual specificity for the DC surface antigen, CD40 and the T cell surface antigen, CD28. The scFv fragments of antibodies directed against these antigens were used to create the αCD40/αCD28-bispecific diabody. A bispecific diabody is a heterodimer formed by non-covalent association of two scFvs consisting of V_H domain from one antibody connected with a short linker to the V_L domain of another antibody (figure 1A/B). The bacterial expression vector pKID_40x28 was constructed and expression of the αCD40/αCD28-bispecific diabody was induced in XL-1 blue E.Coli cells. Presence of the bispecific diabody in the periplasmic fraction of the bacteria and after subsequent purification was confirmed by western blot analysis (figure 2). The predicted molecular weight of both scFv's is 32 kD. The amount of purified protein ranged from 300-600 μg/100 ml culture. As observed by Coomassie Brilliant blue staining purity after affinity chromatography by Ni-beads was suboptimal and estimated at around 10%. To control for impurity proteins isolated from untransformed bacteria were used as a control in the experiments, as described in material and methods.

Increased cluster formation of AML-DC and T cells in presence of αCD40/αCD28-bispecific diabody

To examine if the αCD40/αCD28-bispecific diabody enabled simultaneous binding of AML-DC and T cells, an aggregation assay was performed. Clusters were detected by light microscopy as depicted in figure 3A. Significantly increased cluster formation ($p<0.05$) was observed upon incubation of AML-DC and T cells in the presence of bispecific diabody as compared to control cultures in which AML-DC and T cells were treated with control proteins or were left untreated (figure 3B). Blocking CD40 on AML-DC with the G28-5 antibody prevented AML-DC-T cell clustering in presence of the diabody. Similarly, also CD28 blockade prior to incubation with the diabody prevented AML-DC–T cell

cluster formation. These results thus demonstrate that the αCD40/αCD28-bispecific diabody induces increased AML-DC–T cell interactions through simultaneous and specific targeting and binding of CD40 and CD28.

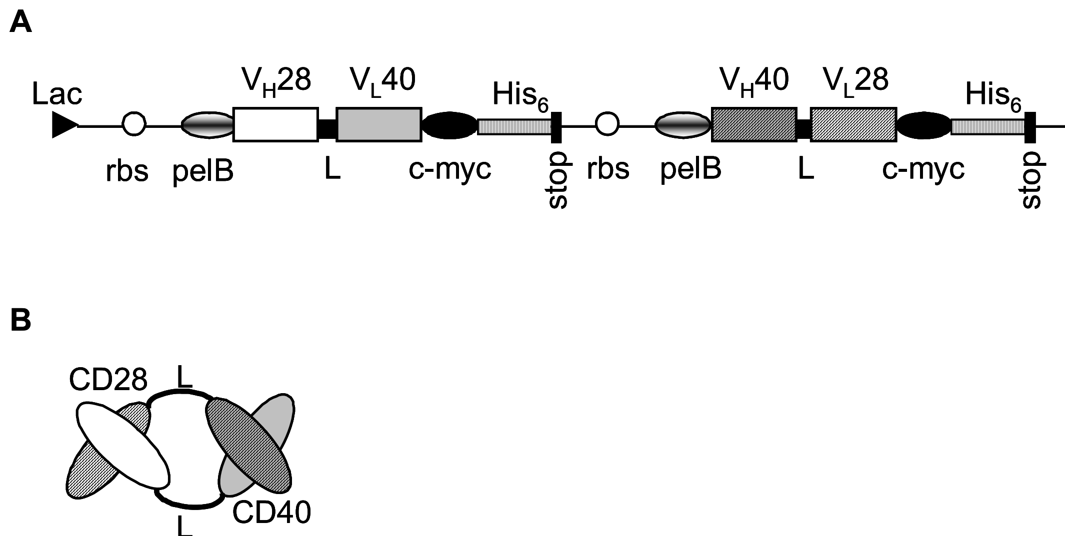


Figure 1. (A) Schematic representation of insert in plasmid pKID_40x28, encoding the αCD40/αCD28-bispecific diabody. Insert is under transcriptional control of the lac promoter and starts behind the pelB leader sequence used for direction of the diabody to the periplasmic fraction. Myc and His_6 tags were included for detection and purification. Locations of ribosomal binding sites (rbs) and stop codons (stop) are depicted for the V_H28/V_L40 and V_H40/V_L28 fusion constructs. (B) Bispecific diabody model shows the noncovalent association of the two resulting scFv hybrids.

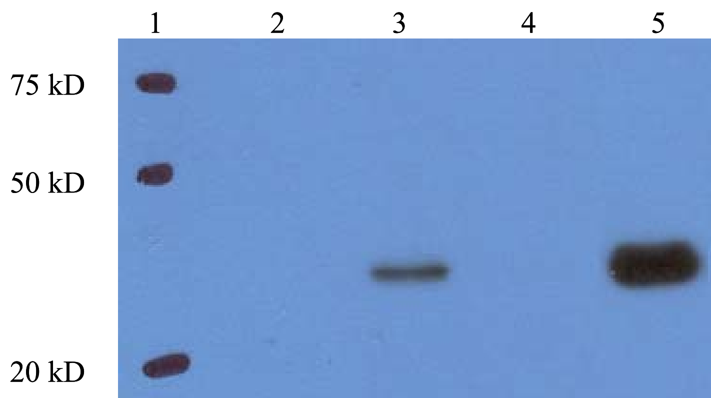


Figure 2. Western blot analysis of periplasmic fractions and αCD40/αCD28-bispecific diabody (BsDb) before and after isolation. Staining was performed using anti-His_6 monoclonal antibody conjugated to horseradish peroxidase. Predicted molecular weight of both scFvs is 32 kD. Lane 1: marker, lane 2: induction of untransformed bacteria (control protein), lane 3: induction of transformed bacteria (αCD40/αCD28 bispecific diabody (BsDb), lane 4: Ni-treated control protein, lane 5: Ni-isolated BsDb.

AML-DC maturation inducing potential of the αCD40/αCD28-bispecific diabody

Since ligation of CD40 is known to induce maturation of DC, the maturation inducing potential of the αCD40/αCD28-bispecific diabody was compared with a standard maturation cocktail of inflammatory cytokines as described in materials and methods, CD40L and G28-5. Figure 4 depicts the MFI of the costimulatory molecules CD80 and CD86 as well as the maturation marker CD83 before and after maturation. The inflammatory cytokine cocktail was used as positive control. The αCD40/αCD28-bispecific diabody was able to induce maturation of AML-DC to a similar extent as CD40L and G28-5, as shown by upregulation of the MFI of the co-stimulatory molecules and the

maturation marker CD83. Incubation with control protein resulted in minimal and significantly less phenotypic maturation induction (see figure 4), excluding the possibility that the observed maturation induction by the diabody might have been caused by contaminating traces of bacterial endotoxins. Thus, incubation with αCD40/αCD28 diabody induces AML-DC maturation as evidenced by up-regulation of CD80, CD86 and CD83.

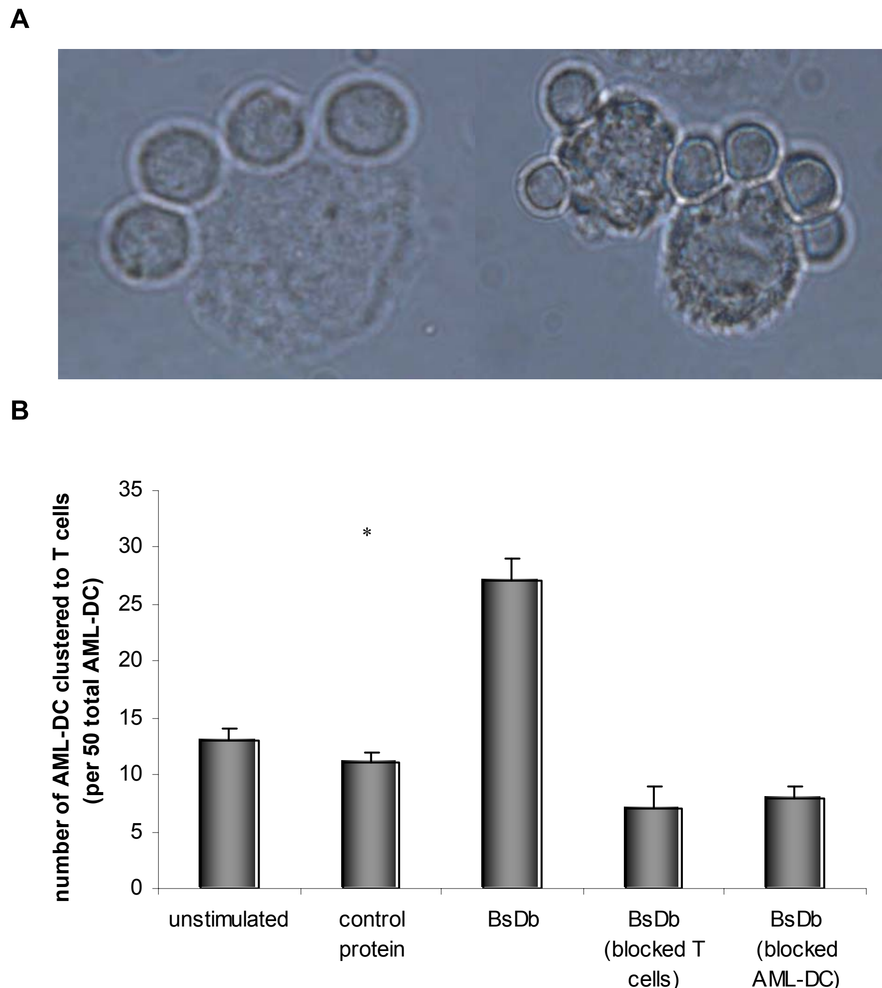


Figure 3. (A) Development of AML-DC - T cell clusters in the presence of the αCD40/αCD28-bispecific diabody as visualized by light microscopy. (Magnification 100x). (B) Clusters of AML-DC and T cells (defined as 2 or more T cells connected to 1 AML-DC) were counted by light microscopy by two independent observers. A total of 50 AML-DC were analyzed and the number of AML-DC forming a complex with T cells were counted in a blinded manner. Mean and standard error of the mean of 3 experiments are shown. * $p<0.05$ as compared to the other cultures

Increased T cell proliferation induced by AML-DC in the presence of the αCD40/αCD28-bispecific diabody

The functional capacity of αCD40/αCD28-bispecific diabody to improve T cell proliferation induced by AML-DC was evaluated by allogeneic MLR. Figure 5 shows that the αCD40/αCD28-bispecific diabody significantly increases T cell proliferation induced by AML-DC as compared to the

unstimulated cocultures, in a dose dependent manner. Separate addition of the anti-CD40 MoAb G28-5 resulted in modestly increased T cell proliferation. An antagonistic CD28 MoAb completely abrogated T cell proliferation (figure 5A). Of note, simultaneous addition of G28-5 and CD28 did not result in increased T cell proliferation, clearly demonstrating the effect of the diabody to be dependent on the resulting cellular cross-linking. Similar results were observed using MoDC (data not shown). These results indicate that αCD40/αCD28-bispecific diabody significantly increases T cell proliferation.

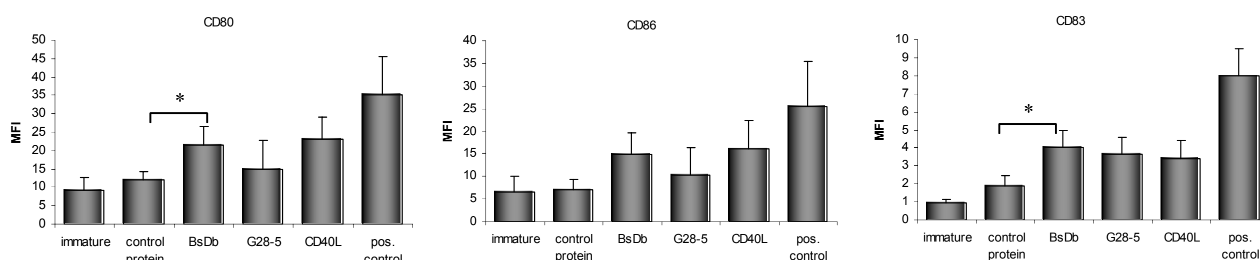


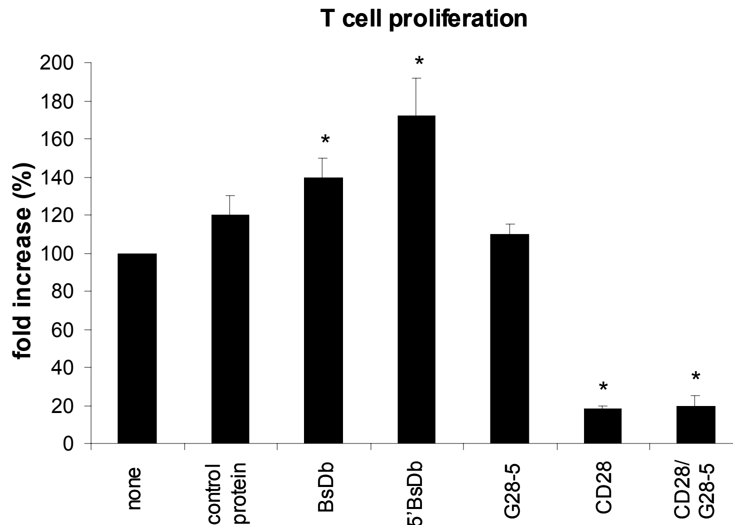
Figure 4. Mean fluorescence index (MFI) of the co-stimulatory molecules CD80 and CD86 and DC maturation marker CD83 before and after maturation. AML-DC maturation in presence of the standard inflammatory cytokine cocktail was used as positive control (pos. control). BsDb: αCD40/αCD28 bispecific diabody. Mean and standard error of the mean of 5 experiments are shown. * $p<0.05$.

The effect of CD40/CD28-mediated cross-linking of AML-DC and $CD8^+$ T cells on the in vitro induction of tumor-specific CTL.

In order to determine the effect of αCD40/αCD28-bispecific diabody-mediated cross-linking of AML-derived DC and $CD8^+$ T cells on the induction efficiency of TAA-specific CTL, DC derived from the HLA-$A2^+$ AML cell line MUTZ-3 were pre-incubated with the αCD40/αCD28-bispecific diabody, loaded with the heteroclitic variant of the aa988 epitope of hTERT (the catalytic subunit of the telomerase complex and known to be an AML-associated TAA), and used as stimulator cells in an HLA-A2-matched allogeneic *in vitro* CTL induction protocol, as described (30). In total nine parallel bulk cultures, at $1x10^6$ $CD8\beta^+$ T cells per culture, were stimulated twice with peptide-loaded MUTZ-3 DC, either pulsed with control protein or the αCD40/αCD28-bispecific diabody. After two DC stimulation rounds, T cells were further expanded by weekly stimulations with peptide-loaded JY cells. From the second round of stimulation, the induction of $hTERT_{988Y}$-specific $CD8^+$ T cells was monitored by tetramer (Tm)-PE/-APC-double staining (see figure 6A). $hTERT_{988Y}$-specific $CD8^+$ T cells could be detected in 5/9 individual cultures when stimulated with DC pulsed with the αCD40/αCD28-bispecific diabody, whereas in only 1/9 individual cultures $hTERT_{988Y}$-specific $CD8^+$ T cells could be detected when stimulated with DC pulsed with the control protein (Tm^+ frequency at a detection threshold of 0.1% of total $CD8^+$ CTL); the highest achieved frequencies, reached after nine *in vitro* stimulation (IVS) rounds, are shown (Figure 6A). To test the generated CTL for functionality, they were enriched by tetramer-guided MACS and expanded. The $hTERT_{988Y}$-specific $CD8^+$ T cells lines were subsequently analyzed for their cytotoxic potential in a CD107a degranulation assay. As shown in figure 6B, both T cells generated with αCD40/αCD28-bispecific diabody-pulsed and with control protein-pulsed DC were able to recognize $hTERT_{988Y}$-loaded JY target cells. Furthermore, avidity analysis of these $CD8^+$ T cell lines on HLA-$A2^+$ JY targets, which were loaded with 10-fold dilutions of $hTERT_{988Y}$ peptide, revealed

both to exhibit high avidity functional T cell recognition, with a half maximal CD107a translocation of > 10 fM peptide (data not shown). These data indicate that the priming efficiency of tumor-specific CTL can be improved by cross-linking AML-DC and T cells via the αCD40/αCD28-bispecific diabody, without affecting functional avidity of the primed CTL.

A.



B.

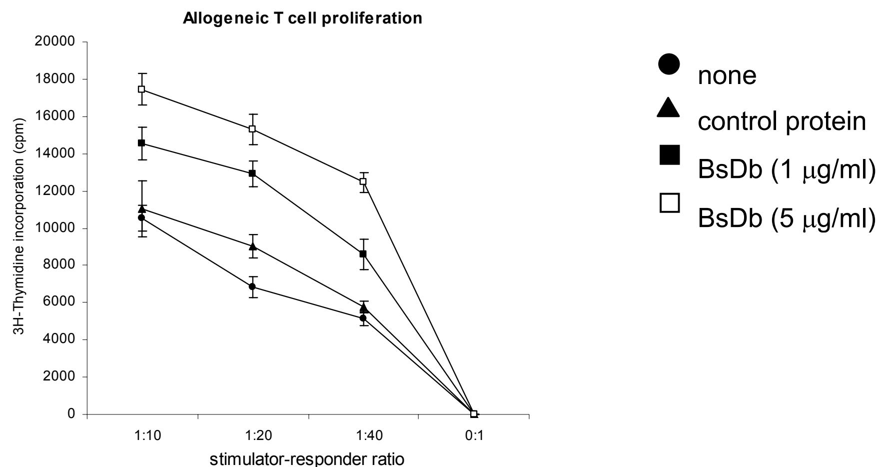


Figure 5. (A) Allogeneic T cells and irradiated AML-DC, at 10:1 ratio, were cocultured for 5 days in the presence of the αCD40/αCD28-bispecific diabody (BsDb) (1 (BsDb) or 5 μg/ml (5*BsDb)), control protein (1 μg/ml), G28-5 (1 μg/ml), CD28 (2 μg/ml) or a combination of G28-5 and CD28. Untreated cocultures were taken as a reference. T cell proliferation was measured by [^{3}H] thymidine incorporation. Mean percentages (related to proliferation found for the control condition without added protein, BsDb, or mAbs-set at 100%) and standard error of the mean of 5 independent experiments are shown. * p<0.05 as compared to untreated cocultures. (B) A representative example of a MLR with allogeneic T cells and irradiated AML-DC at different stimulator/responder (S/R) ratio's. Mean counts per minute (cpm) and standard error of the mean of triplicate cultures are shown.

Discussion

In this study we report the successful construction of a novel bispecific diabody, simultaneously and agonistically targeting CD40 on DC and CD28 on T cells, and demonstrate specific binding of the

diabody to its target antigens and its ability to increase AML-DC–T cell cluster formation, AML-DC-induced T cell proliferation, as well as the induction of high avidity tumor-specific CTL. Furthermore, the diabody is capable of increasing AML-DC maturation status to a similar extent as CD40L.

T cells require both primary and costimulatory signals for optimal activation. DC, serving as central mediators of T cell-based immunity, are especially important for T cell activation. The costimulatory molecules CD80 and CD86, expressed by DC, interact with CD28 on naïve T cells. Costimulation by CD28 drives T cell cycle progression, induces T cell differentiation, promotes T cell survival and amplifies membrane proximal signaling induced by TCR ligation (34). In view of this CD28 costimulation has been intensively investigated and proven to contribute to anti-tumor T cell responses (35-38).

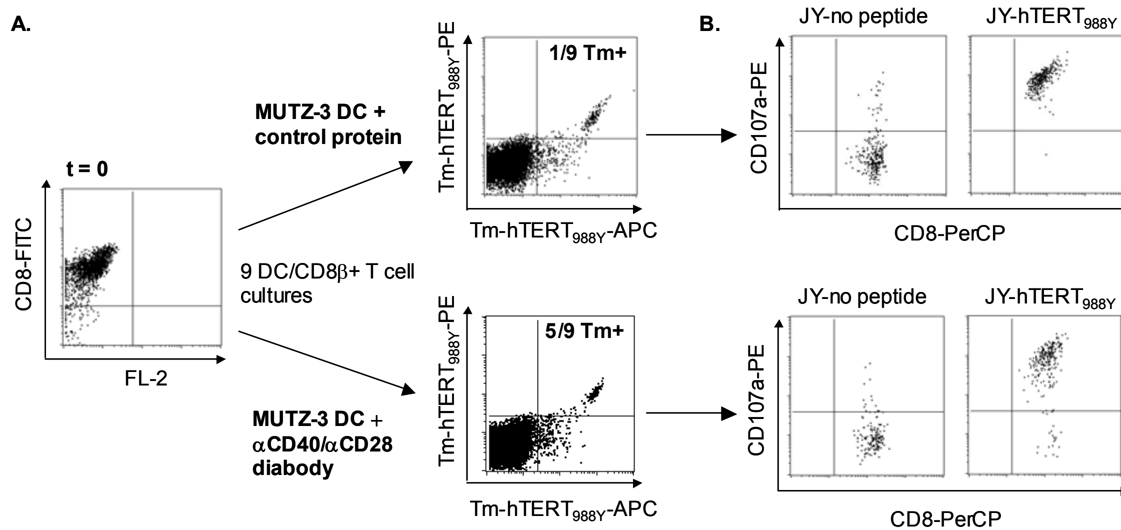


Figure 6. Flow cytometric HLA-A2$^+$ tetramer (Tm) binding and functional analysis of CD8β$^+$ hTERT$_{988Y}$-specific CTL primed by MUTZ-3 AML DC, pre-incubated with a control protein or the αCD40/αCD28-diabody. From day 0 (t=0) magnetic bead-isolated CD8β$^+$ CTL precursors were stimulated repeatedly with either αCD40/αCD28-diabody- or control protein-pulsed hTERT$_{988Y}$-presenting MUTZ-3 AML DC. (A) Flow cytometric FITC-labeled CD8 staining on isolated CD8β$^+$ CTL precursors and PE-/APC-labeled tetramer (Tm) analysis on primed and expanded CD8β$^+$ CTL precursors. Staining with both PE- and APC-labeled Tm was performed to exclude false positive staining by single Tm. Representative results for live, propidium iodide-negative cells are shown. Cultures were scored positive when percentage of tetramer-positive cells exceeded 0.1 % of live cells. Number of hTERT$_{988Y}$-Tm$^+$ positive cultures in αCD40/αCD28-diabody- or control protein-stimulated cultures (of nine cultures started) is depicted in the upper right quadrant. (B) Functional activity of the generated hTERT$_{988Y}$-specific CD8$^+$ T cell lines was determined by CD107a degranulation assay. Target cells used were either unloaded HLA-A2$^+$ JY cells or JY cells loaded with the hTERT$_{988Y}$ peptide. CD107a$^+$ cells shown were gated for live tetramer-hTERT$_{988Y}$$^+$/CD8$^+$ T cells.

Simultaneous targeting of tumor cells and T cells through the use of a bispecific diabody has been shown to increase effector immune functions (17,39,40). We now demonstrate that facilitated interaction of DC and T cells by bridging both cell types with a bispecific diabody, possibly strengthening the immunological synapse, contributes to increased immune responses. Chosen targets were CD28 and CD40 because both antigens are highly expressed by naïve T cells and AML-DC, respectively. Since AML-DC display a heterogeneous expression of the costimulatory molecules CD80 and CD86, bypassing the need for costimulation via CD80 and CD86 by directly and

agonistically targeting CD28 is an important advantage of the constructed bispecific diabody (3,4). Apart from interacting with CD28, CD80 and CD86 are the natural ligands for the inhibitory co-stimulatory molecule CTLA-4, thereby down-regulating the immune response. It could be speculated that targeting CD28 by the bispecific diabody might result in an increased number of CD80 and CD86 molecules available to interact with CTLA-4, inducing an increased down-regulation of T cell responses. However, since the diabody will be employed to target naïve or central memory T cells recirculating through lymph nodes draining the intended (intradermal) AML-DC vaccination site and since CTLA-4 is only expressed upon T cell activation (41), it does not seem likely that this situation will arise.

Recently, the unfortunate results of a phase 1 clinical trial of the superagonistic monoclonal CD28 antibody, TGN1412, drew a lot of attention (42). Intravenous administration of this antibody evoked a critical cytokine storm in all 6 healthy volunteers. TGN1412 bypasses the requirement for TCR triggering and activates T cells irrespective of their TCR specificity. In contrast to TGN1412 the constructed bispecific diabody described here is directed against both CD40 and CD28 thereby aiming at the specific interaction of AML-DC and T cells in the context of the MHC and TCR complex. Prior to intradermal administration AML-DC would be incubated with the diabody and repeatedly washed to eliminate unbound diabody, thus precluding the wide-spread and pervasive systemic effects that were achieved by intravenous infusion of the TGN1412 superagonist. Of course further studies are required in order to determine the affinity of the bispecific diabody for both ligands to further show how this compared to the observed high affinity of the TGN1412 antibody.

Besides T cells also neutrophils express CD28. Cross-linking may result in the release of IFN-γ by these neutrophils and the induction of a T cell chemotactic factor thereby possibly modulating T cell responses towards a T helper 1 subtype (43). Additionally, interaction between DC and activated neutrophils is known to induce DC maturation that enables these DCs to trigger strong T cell proliferation and T helper type 1 polarization of T cells (44). To what extent these effects will be provoked and strenghtened by the bispecific diabody will be an interesting subject of future research.

The bispecific diabody also agonistically targets CD40 thereby offering the advantage of inducing an enhanced DC activation status, since it is known that CD40 ligation induces DC maturation and activation (8,45-48). Indeed the αCD40/αCD28-bispecific diabody is capable of inducing DC maturation to a similar extent as CD40L.

Blocking experiments in the aggregation assay demonstrated specific binding to CD40 and CD28. These experiments also showed increased cluster formation upon incubation of AML-DC and T cells with the bispecific diabody as compared to control cultures. Specific binding to CD28 was also demonstrated by the prevention of inhibited T cell proliferation upon CD28 blockade, by prior incubation with the bispecific antibody (data not shown).

Targeting CD28 and CD40 by the constructed bispecific diabody can increase the strength and duration of interaction between DC and T cells that occurs in the immunological synapse. Indeed, enhanced T cell responsiveness was observed upon co-culturing AML-DC and allogeneic T cells in the presence of the αCD40/αCD28-bispecific diabody resulting in increased T cell proliferation. In addition, the detection of an increased frequency of cultures with tetramer positive CTL in the

αCD40/αCD28-bispecific diabody stimulated T cell cultures as compared to control protein-stimulated T cell cultures, is highly suggestive of an improved induction efficiency of tumor-specific CTL by cross-linking AML-DC and T cells via the αCD40/αCD28-bispecific diabody.

For clinical purposes it is important that the αCD40/αCD28-bispecific diabody does not contain any bacterial components. Since the purification by Ni-mediated affinity chromatography did not result in completely pure products, we are currently developing new strategies to purify the bispecific diabody, such as size exclusion chromatography. For *in vitro* experiments XL-1 blue-derived bacterial proteins were taken as control for the bacterial content contaminating the αCD40/αCD28- bispecific diabody. These proteins did not bind to target antigens nor induced T cell proliferation. They showed minimal maturation induction, indicating that the observed maturation induction by the αCD40/αCD28-bispecific diabody is due to its agonistic effects on AML-DC and T cells, through simultaneous CD40- and CD28-mediated activation, rather than to lingering endotoxin contaminants.

T cell stimulating capacity of αCD40/αCD28- bispecific diabody was evaluated in a model using AML-DC as antigen presenting cells. However, it is interesting to speculate on a broader application of the diabody as adjuvant therapy. For example, co-injection of the bispecific diabody with monocyte-derived DC loaded with tumor-specific antigens could represent a powerful vaccination strategy, increasing the spectrum to a wide range of malignancies.

To summarize, the newly developed αCD40/αCD28-bispecific diabody is capable of augmenting T cell responses induced by AML-DC. The dual role of both CD40 and CD28 as antigenic targets as well as inducers of DC maturation and T cell activation respectively, might effectively enhance AML-DC-induced T cell immunity *in vivo*. The αCD40/αCD28- bispecific diabody may thereby provide a powerful therapeutic tool necessary to strengthen immune responses induced by DC, presenting tumor-specific antigens.

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