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Highly efficient antigen targeting to M-DC8$^+$ dendritic cells via FcγRIII/CD16-specific antibody conjugates

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Abstract

Conjugates of peptide antigens with antibodies specifically recognizing surface molecules on dendritic cells (DC) represent an attractive approach to target antigens to antigen-presenting cells (APC) for the induction of specific T cell responses. The present study evaluates the potential of M-DC8$^+$ DC, a sub-population of professional APC in the blood, for an antibody-based vaccination strategy. We prepared, by chemical cross-linking, conjugates of peptide model antigens with antibodies directed against different cell surface molecules of DC. Antigen–peptide conjugates using an anti-CD16 (FcγRIII) antibody were most potent in inducing *in vitro* activation of a specific CD4$^+$ T cell response. They were at least 300 times more efficient than two other antibody–antigen conjugates and ~500 times more efficient than unconjugated antigen peptides. Our data demonstrate that specific antigen targeting via CD16 on M-DC8$^+$ DC is a promising vaccination approach for the efficient induction of specific CD4$^+$ T cell responses *ex vivo*, and perhaps *in vivo*.

Introduction

Dendritic cells (DC), as the most potent antigen-presenting cells (APC), are currently tested in various vaccination approaches as natural adjuvants. DC do not only activate quiescent, memory and naive CD4$^+$ T lymphocytes (1), but have also been shown to cross-present exogenous antigen to CD8$^+$ T cells in the context of MHC class I (2, 3). First clinical trials of human DC vaccination for cancer immunotherapy of e.g. melanoma, prostate cancer and non-Hodgkin lymphoma generated encouraging preliminary results by using DC pulsed *ex vivo* with different tumor-specific peptides (4). Most clinical studies focus on treatment of melanoma patients because well-defined tumor-associated antigen-derived peptides like MelanA and MAGE3 are available. Other approaches use DC pulsed with tumor lysate, DC transfected with cDNA or mRNA encoding tumor-relevant antigens (5). However, all these vaccination approaches require the isolation of autologous DC or their precursors from the individual patients followed by *ex vivo* differentiation, antigen loading and maturation of DC prior to re-injection into the patient (4, 6).

As the requirement of expensive individual treatment limits the potential of DC vaccination for broad clinical applications, direct antigen delivery to DC *in vivo* by antigen linked to DC-specific antibodies represents an attractive alternative to the currently pursued DC vaccination approaches. Vaccines based on direct antigen targeting to DC are well defined due to relatively few influencing parameters and might therefore allow a better prediction of dose response and treatment benefit for an individual patient. Strategies for specific antigen targeting to DC for *in vivo* vaccination purposes depend on the availability of surface markers on DC that mediate efficient antigen uptake. In contrast to the murine system where specific antigen targeting to DC by CD11c antibodies led to efficient induction of a humoral immune response (7), there is still a lack of human DC-specific markers which have been proven to be useful for antigen-targeting purposes. Receptors for the constant domain of IgG (FcγR) have been shown to play a major role in the immune defense. In addition, there is abundant evidence that complexation of antigen with IgG leads to efficient antigen uptake by FcγR. Studies with the immune-complexed model antigen ovalbumin in the murine system (8, 9) and more recently human studies with immune complexes as well as antibody-coated tumor cells (10, 11) indicate an involvement of FcγR in cross-presentation.

M-DC8$^+$ cells have recently been identified as a new sub-population of DC in human blood representing ~1% of PBMC (12). In contrast to the other DC sub-populations, DC1 and

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DC2, which show an even lower frequency in human blood, M-DC8$^+$ cells express high levels of FcγRIII (CD16) but lack the expression of FcγRI (CD64) (12, 13). Accordingly, FcγRIII lends itself as a target for antigen-specific T cell activation through M-DC8$^+$ DC. The new mAb D-DC8.3, which recognizes a yet unknown surface marker on ~50% of M-DC8$^+$ cells, was also considered for DC-specific antigen targeting.

In the present study, we evaluated the use of monoclonal anti-CD16 and D-DC8.3 antibodies to deliver model antigens to M-DC8$^+$ DC. By generation of different antibody–antigen conjugates, we were able to analyze and compare the targeting efficiency of anti-CD16, D-DC8.3, a control antibody and unconjugated antigen with respect to MHC class I- and MHC class II-restricted antigen presentation through M-DC8$^+$ cells. We could demonstrate that CD16/FcγRIII-mediated antigen uptake by M-DC8$^+$ cells induces a very potent activation of CD4$^+$ T cells while cross-presentation of antigen by M-DC8$^+$ cells on MHC class I seemed to be FcγRIII independent. We discuss the potential of this vaccination strategy in comparison to existing approaches and for *in vivo* application as a vaccine.

Methods

Media and reagents

RPMI 1640 was used throughout as cell culture medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids (aa), 1% pyruvate, 50 μg ml^{-1} kanamycin (GIBCO, Grand Island, NY, USA), 5 × 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany) and 10% FCS (Hyclone Laboratories, Inc., Logan, UT, USA). Human recombinant IL-4 was produced by PCR cloning and expression in a myeloma-based expression system (14). Granulocyte macrophage colony-stimulating factor (GM-CSF) (Leucomax) was purchased from Sandoz (Basel, Switzerland).

Isolation and culture of M-DC8$^+$ cells

Isolation of M-DC8$^+$ cells was performed as previously described (13). Briefly, PBMC from an HLA-matched donor were incubated for 30 min with undiluted supernatant of the M-DC8 hybridoma (containing ~10 μg ml^{-1} antibody). After washing, M-DC8$^+$ cells were isolated using rat anti-mouse IgM microbeads and an LS$^+$ separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). If necessary, a second purification step on an MS$^+$ separation column was followed to obtain a purity of cells >95%. Immature M-DC8$^+$ DC were obtained by a 4- to 5-day culture in RPMI/10% FCS supplemented with 50 ng ml^{-1} GM-CSF and 1000 U ml^{-1} human recombinant IL-4.

Antibodies and flow cytometry

The D-DC8.3 hybridoma was kindly provided by E. Rieber (University of Dresden, Germany). D-DC8.3 antibody was purified from hybridoma supernatant by affinity chromatography using a sepharose gel coupled to anti-mouse IgG. Murine anti-CD16/FcγRIII (clone 3G8) was purchased from PharMingen (San Diego, CA, USA), and a mouse IgG$_1$ antibody (Iso-IgG) used as an isotype control was obtained from Southern Biotechnology Associates (Birmingham, AL, USA). The mouse anti-human NKG2D antibody was purified from a hybridoma supernatant using a two-step purification process (thiophylic interaction chromatography and gel filtration). For flow cytometric analysis, the following antibodies were used: anti-CD11c-FITC (DAKO Corporation, Carpinateria, CA, USA), anti-CD3-APC (Caltag Laboratories, Burlingame, CA, USA), anti-CD25-PE, anti-CD69-PE (both from PharMingen) and FITC- and PE-conjugated goat anti-mouse IgG secondary antibodies (Southern Biotechnology Associates). The PE-conjugated peptide MHC tetramers were a gift from D. Busch (Technical University of Munich, Germany). Stained cells were analyzed on FACSCalibur cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with Cellquest software (Becton Dickinson). Dead cells were excluded by propidium iodide (PI) staining.

Antibody–antigen conjugates

The following antigenic peptides for conjugate preparation were synthesized by Jerini (Berlin, Germany): tetanus toxoid (TT) peptide (5,6-carboxyfluoresceine-GGGQYIKANSFIGITEGGGC) derived from TT aa 830–843, non-structural protein 3 (NS3) peptide (5,6-carboxyfluoresceine-KGGGYKVLVLNPSVAATGGGC) from the NS3 protein of hepatitis C virus aa 1248–1261 and Her2/neu peptide (5,6-carboxyfluoresceine-KKIFGSLAFLPESC) derived from human Her2/neu aa 369–377. Each antigenic peptide consisted of a T cell epitope (underlined) flanked by additional aa. The MHC class II-restricted TT and NS3 epitopes are both presented by HLA class II DR11 (15, 16); the MHC class I-restricted Her2/neu epitope is presented on HLA-A*0201 (17). For preparation of conjugates, antigenic peptides were covalently linked to anti-CD16, D-DC8.3 and isotype control antibodies. All peptides had C-terminal cysteine residues for conjugation. A carboxyfluoresceine moiety was added for detection purposes at the N-terminus and a free sulfhydryl group in a cysteine residue for conjugation at the C-terminus.

The antibodies were dialyzed against a buffer containing 0.1 m NaCl, 0.05 m boric acid and 0.01 m EDTA, pH 7.2. To generate antibody–peptide conjugates, sulfhydryl-reactive maleimide groups were introduced into the antibody (anti-CD16, D-DC8.3 and Iso-IgG) by a 1-h incubation with a 60-fold molar excess of the cross-linker sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (Pierce, Rockford, IL, USA) pre-dissolved in water-free dimethylsulfoxide. After activation, uncoupled cross-linker was removed by concentration in an 'Ultrafree' spin filter unit with a 10-kDa molecular weight cut-off (Millipore, Billerica, MA, USA). The concentrate was filled up to 90 μl with the above-described boric acid/EDTA buffer, pH 7.2. The activated antibodies were mixed with 10 μl antigenic peptide solution (10 mg ml^{-1} in water-free dimethylsulfoxide), followed by a 1-h incubation at room temperature. Antibody–peptide conjugates were purified by size-exclusion chromatography with P60 fine cross-linked polyacrylamide beads (Biorad, Hercules, CA, USA) to remove free antigenic peptide. The concentration of conjugates was measured by ELISA using an isotype-matched antibody as the reference. The molar ratio of peptide bound to antibody was calculated by fluorochrome-to-protein ratio (F/P) analysis (18, 19) after determination of absorptions at 280 and 493 nm using the following equations:

$$\text{fluorescein-coupled IgG concentration (FL IgG conc.)}\ (\text{mg ml}^{-1}) = (A_{280\,nm} - 0.35 \times A_{493\,nm})/1.4,$$

where 1.4 is the optical density for 1 mg ml^{-1} IgG. The molar ratio (F/P) can then be calculated based on a molar extinction coefficient of 73 000 for the fluorescein group (18, 19):

$$F/P = (A_{493\,nm}/73\,000) \times (150\,000/\text{FL IgG concentration}).$$

All conjugates were normalized to statistically contain one antigen molecule per antibody.

Conjugates of antibodies with full-length protein antigen were prepared using a commercially available protein–protein cross-linking kit (Pierce). The complete extracellular domain (ECD) of human Her2/neu was expressed in CHO cells and the purified protein was chemically cross-linked either with the anti-CD16 antibody or the isotype control. In addition, we generated control conjugates using the ECD of Epidermal Growth Factor receptor (EGFR) as a homologous but irrelevant antigen of comparable size. The cell lines producing soluble Her2/neu and EGFR were kindly provided by Tobias Raum. Conjugates were separated from free antigen, antibody or multimeric conjugates by gel filtration and the concentrations of the conjugates were determined by absorbance at 280 nm.

T cell activation studies

M-DC8$^+$ cells were isolated from PBMC of an HLA-matched donor as described above. For analysis of MHC class II presentation, 25 000 M-DC8$^+$ cells were mixed with 10 000 antigen-specific CD4$^+$ cells per well and cultivated with titrated amounts of antibody–antigen conjugate in a final volume of 200 μl RPMI/10% FCS at 37°C for 14 h. T cell activation was analyzed by measuring CD25 expression on a flow cytometer after staining of cells with anti-CD25-PE, anti-CD11c-FITC (M-DC8$^+$ cells) and anti-CD3-APC (T cells) antibodies as well as PI (for exclusion of dead cells). The TT-specific CD4$^+$ T cell clone was kindly provided by A. Lanzavecchia (Bellinzona, Switzerland) (15) and the NS3-specific CD4$^+$ T cell clone was a kind gift of G. Pape (University of Munich, Germany) (16). For analysis of cross-presentation, 25 000 M-DC8$^+$ cells were incubated with titrated amounts of antibody–antigen conjugate at 37°C for 14 h, washed twice with medium to remove unbound conjugate before adding 10 000 Her2/neu-specific CD8$^+$ T cells (a kind gift of H. Bernhard, Technical University of Munich, Germany) (17) in a final volume 200 μl per well. After 24 h, T cell activation was determined by flow cytometric analysis using a PE-conjugated anti-CD69 antibody instead of anti-CD25-PE.

Results

Target structures on M-DC8$^+$ cells

FcγR have been shown to be involved in efficient antigen uptake and might therefore represent a potential structure for antigen targeting on DC. M-DC8$^+$ DC showed very high surface expression of FcγRIII/CD16 as analyzed by flow cytometry (Fig. 1, left panel). In addition, ~50% of the M-DC8-positive cells are specifically recognized by a new mAb termed D-DC8.3 (Fig. 1, right panel). This antibody stains a cell population with nearly identical properties as the M-DC8$^+$ cells, especially regarding the expression of MHC molecules and co-stimulatory molecules as revealed by flow cytometric analysis (data not shown). We therefore decided to use both a mAb against CD16 (clone 3G8) as well as the D-DC8.3 antibody for the generation of conjugates with model peptide antigens. An irrelevant isotype-matched antibody designated Iso-IgG was included as control.

FcγR III/CD16-specific conjugates enhance TT-specific activation of CD4$^+$ T cells by M-DC8$^+$ cells

Freshly isolated M-DC8$^+$ cells from a human HLA-matched donor were mixed with TT-specific CD4$^+$ T cells and incubated with titrated amounts of anti-CD16–TT peptide conjugate, D-DC8.3–TT peptide conjugate or control Iso-IgG–TT peptide conjugate for 14 h. Prior to the assay, functional binding of the antibody–peptide conjugates on human PBMC was confirmed by flow cytometry [Fig. 2 (anti-CD16) and data not shown (D-DC8.3)].

T cell activation was measured by flow cytometric analysis of CD25 expression on T cells. The dose–response curves in Fig. 3(A) showed that <3 ng ml^{-1} of anti-CD16–TT peptide

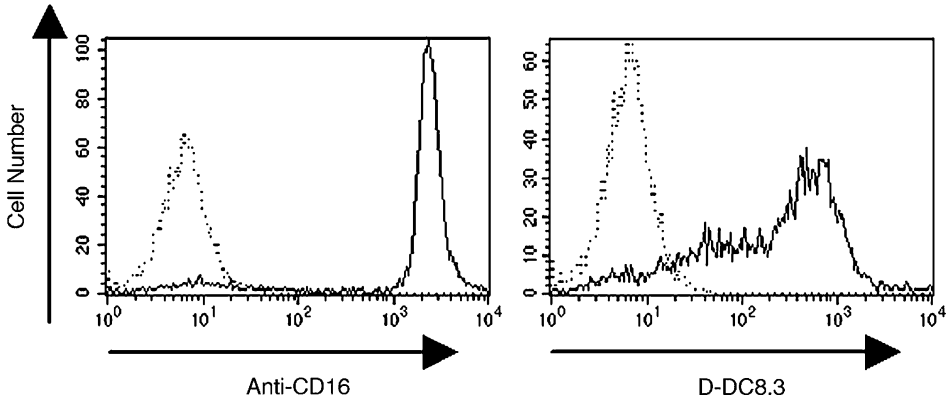

Fig. 1. Specificity of anti-CD16 and D-DC8.3 antibodies for M-DC8-positive cells. Freshly isolated M-DC8-positive cells from human blood were stained with anti-CD16 (left panel, solid line) or D-DC8.3 antibodies (right panel, solid line). Dotted lines represent unstained M-DC8-positive cells as a control.

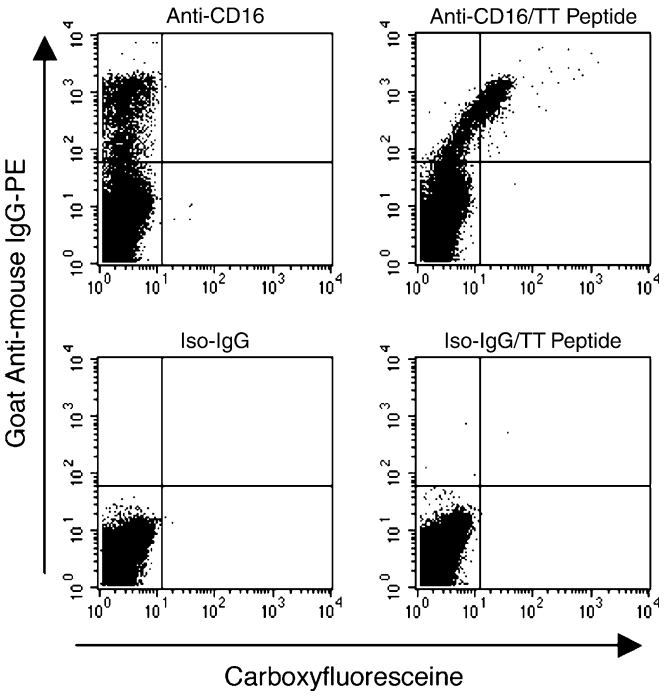

Fig. 2. Binding analysis of antibody–peptide conjugates to human PBMC by flow cytometry. Cells were incubated with conjugates or complete antibody followed by staining with a PE-conjugated anti-mouse antibody for detection. Conjugates consist of an antibody (anti-CD16 and an isotype-matched control antibody referred to as Iso-IgG) chemically linked to a peptide including a T cell epitope derived from TT and a carboxyfluoresceine. Dead cells were excluded by PI staining. The anti-CD16/peptide conjugate bound the NK cell population of PBMC with the same efficiency as the unconjugated anti-CD16 antibody (upper panels), while both Iso-IgG control antibody and the respective control conjugate did not bind to PBMC (lower panels).

conjugate (2×10^{-11} M of epitope) was sufficient to induce half-maximum activation of T cells. D-DC8.3–TT peptide conjugate [half maximal effective dose (ED_{50}) = 300 ng ml^{-1}; 2×10^{-9} M of epitope] and Iso-IgG–TT peptide conjugate (ED_{50} = 1 μg ml^{-1}; 6.67×10^{-9} M of epitope) had an efficiency that was >100 times lower than that of the anti-CD16–TT peptide conjugate. Despite the fact that free antigenic TT peptide can bind to MHC class II without processing, unconjugated TT peptide ($ED_{50} = 2 \times 10^{-9}$ M of epitope) was ~500 times less effective on a molar basis in T cell activation than the anti-CD16-coupled TT peptide. Likewise, mixtures of antibodies and antigenic TT peptide in equimolar ratios did not elicit a T cell response exceeding that of the TT peptide alone (Fig. 3A). Physical linkage between T cell epitope and targeting antibody was thus required for highly efficient T cell activation. Similar results were obtained using antibody–NS3 peptide conjugates and NS3-specific $CD4^+$ T cells (data not shown). The following efficiencies of T cell activation were obtained: anti-CD16–NS3 peptide conjugate: $ED_{50} < 1$ ng ml^{-1}; D-DC8.3–NS3 peptide conjugate: ED_{50} = 100 ng ml^{-1} and Iso-IgG–NS3 peptide conjugate: ED_{50} = 300 ng ml^{-1}.

The need of APC for specific T cell activation was demonstrated by the fact that 10 μg ml^{-1} (6.67×10^{-8} M of epitope) of antibody–antigen conjugates could not induce

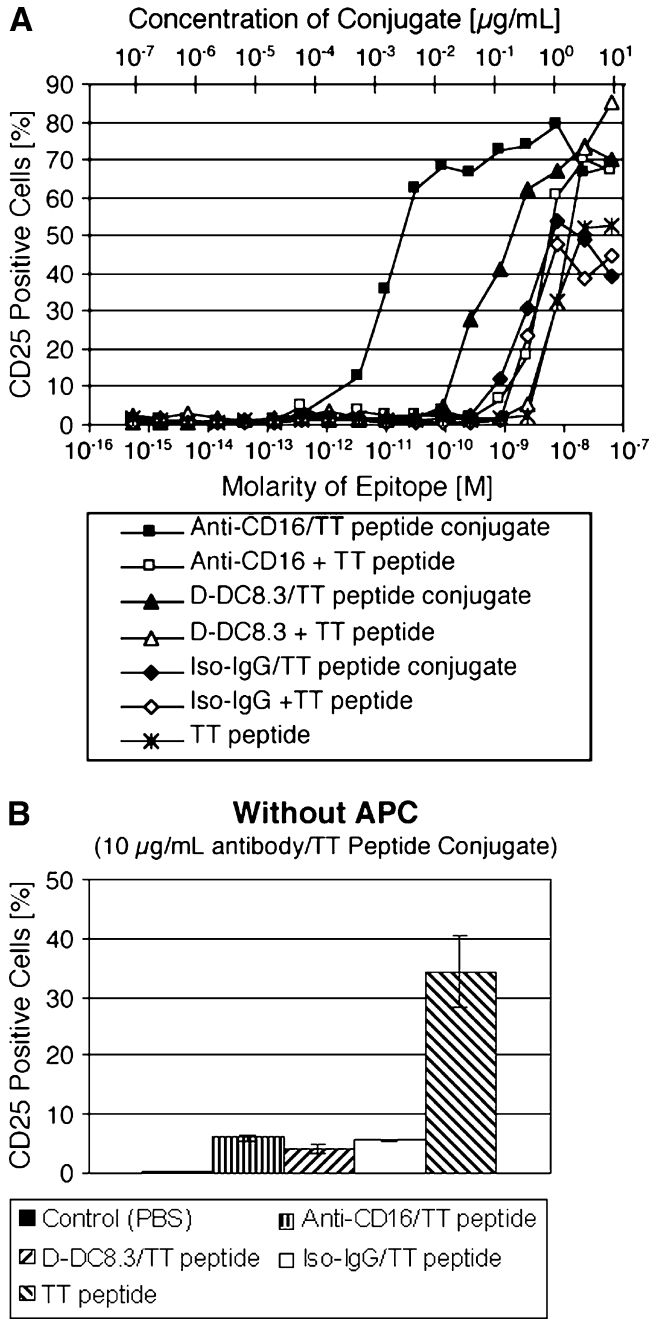

Fig. 3. Anti-CD16/peptide conjugates efficiently deliver T cell epitopes to M-DC8-positive cells. (A) Freshly isolated M-DC8-positive cells and peptide-specific T cells were incubated with titrated amounts of antibody–peptide conjugate, a mixture of peptide and antibody in equivalent molar concentrations or non-conjugated peptide for 14 h. T cell activation was determined by flow cytometric analysis of CD25 expression. Dead cells were excluded by PI staining and a gate was set for T cells based on the expression of CD3 (T cells) and CD11c (M-DC8-positive cells). The result is representative for three independent experiments. (B) Effect of antibody/peptide conjugates on T cells without APC. TT-specific T cells were incubated with 10 μg ml^{-1} of antibody/TT peptide conjugate or TT peptide in equivalent molar concentrations for 14 h. The percentage of CD25-positive cells was determined by flow cytometric analysis. As a negative control, T cells were incubated with PBS instead of conjugate. For maximal stimulation, 5 μg ml^{-1} of TT peptide was used. The data shown are a representative for three independent experiments.

T cell activation in the absence of M-DC8$^+$ cells (Fig. 3B). In contrast, the same amount of free TT peptide at a concentration of 6.67×10^{-8} M activated ~30% of T cells. This can be explained by direct binding of the TT peptide to MHC class II expressed by a minority of pre-activated T cells.

To address the question whether IgG in human serum might inhibit antigen targeting by antibody–peptide conjugates, M-DC8$^+$ cells were pre-incubated with 2.7 mg ml^{-1} of human IgG or 20% human serum prior to the addition of conjugates. After a 1-h incubation, unbound conjugates were removed by washing and the M-DC8$^+$ cells were cultivated with TT peptide-specific T cells for 14 h. Our data indicate that blocking of FcγR by pre-incubation with human serum or IgG did not significantly influence the efficiency of the anti-CD16–TT peptide conjugate in T cell activation (data not shown), although the 3G8 anti-CD16 antibody used was reported to recognize an epitope in the vicinity of the IgG-binding site of CD16 (20, 21).

Immature DC are less suited for antigen targeting than freshly isolated M-DC8⁺ cells

Immature DC are the most efficient cell population regarding antigen uptake (3). Upon culturing M-DC8$^+$ cells in medium containing 50 ng ml^{-1} GM-CSF and 1000 U ml^{-1} IL-4 for 4–5 days, cells developed the phenotype of immature DC (13). If cultured M-DC8$^+$ cells were mixed with TT-specific CD4$^+$ T cells and incubated with titrated amounts of different antibody–antigen conjugates, the T cell activation induced by anti-CD16–TT peptide conjugate was reduced (Fig. 4; ED_{50} = 8 ng ml^{-1}) compared with that induced by freshly isolated M-DC8$^+$ cells (ED_{50} = 3 ng ml^{-1}; see Fig. 3A). This can be explained by the reduction of CD16 surface expression during cultivation of M-DC8$^+$ cells (13). Moreover, the slight shift of the ED_{50} values to lower concentrations for the D-DC8.3–TT peptide conjugate (ED_{50} = 100 ng ml^{-1}) as well as for the Iso-IgG–TT peptide conjugate (ED_{50} = 100 ng ml^{-1}) may indicate an expected increase in the general efficiency of antigen uptake by immature DC. The small differences in ED_{50} values seem to be of minor importance compared with the observation that the percentage of activated T cells is approximately doubled when using freshly isolated M-DC8$^+$ cells instead of cultured M-DC8$^+$ DC. Our data show that freshly isolated M-DC8$^+$ cells are more suitable for CD16-mediated antigen targeting than the immature DC obtained after culturing with cytokines.

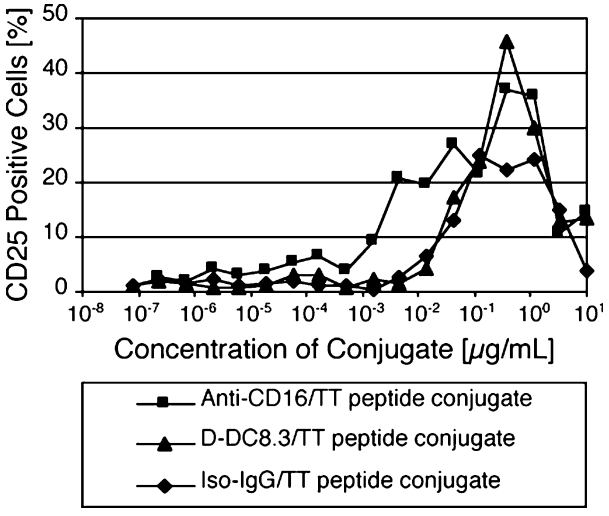

Fig. 4. T cell stimulation induced by cultured M-DC8-positive cells. Freshly isolated M-DC8-positive cells were cultured for 4 days in complete medium with 50 ng ml^{-1} GM-CSF and 1000 U ml^{-1} IL-4 for differentiation into DC with an immature phenotype. M-DC8 DC were co-cultivated with TT-specific T cells and titrated amounts of antibody/TT peptide conjugates for 14 h before the determination of T cell activation by flow cytometric analysis. Experiments were performed as for Fig. 3. The result is representative for two experiments.

M-DC8⁺ cells do not efficiently cross-present after CD16-mediated antigen uptake

To assess the ability of M-DC8$^+$ cells to cross-present antigen on MHC class I after CD16-mediated uptake, a sensitive assay measuring activation of a high-affinity CD8$^+$ T cell clone specific for a defined peptide epitope of human Her2/neu was used. Prior to the assay, binding of the peptide conjugates to human PBMC was confirmed by flow cytometry (Fig. 5A). Freshly isolated M-DC8$^+$ cells of an HLA-matched donor were pulsed with titrated amounts of antibody–Her2/neu conjugates for 14 h before removal of unbound conjugates by two washing steps. Her2/neu-specific CD8$^+$ T cells were added and CD69 expression of T cells was analyzed by flow cytometry 24 h later. Targeting of Her2/neu peptide to M-DC8$^+$ cells by either anti-CD16, D-DC8.3 or the Iso-IgG–Her2/neu peptide conjugate did not detectably induce CD8 T cell activation (Fig. 6A). If T cells were co-stimulated with anti-NKG2D antibodies during co-cultivation with APC in order to increase assay sensitivity, similar results were obtained (data not shown). As the length of CD8 T cell epitopes is more restricted than that of peptides binding to MHC class II molecules (22), the observed lack of CD8 T cell activation could be due to incorrect or deficient peptide processing. To exclude this possibility, we tested additional conjugates with full-length protein antigen in order to maintain the natural context for processing. Prior to the assay, the structural integrity and binding activity of the antibody–protein conjugates were confirmed by flow cytometry using anti-Her2/neu or anti-EGFR antibodies (Fig. 5B) and the specificity of the Her2/neu-specific CD8$^+$ T cell clone was controlled by staining with PE-conjugated Her2/neu-specific MHC class I tetramers (Fig. 5C). However, in the corresponding T cell activation experiments, only a very weak dose-dependent CD69 up-regulation and no anti-CD16-mediated enhancement of cross-presentation could be observed (Fig. 6B). The general functionality of the experimental setting was confirmed by the observation that M-DC8$^+$ cells pulsed with high concentrations of unconjugated Her2/neu ECD protein induced specific CD69 activation of Her2/neu-specific T cells co-stimulated with 100 μg ml^{-1} anti-NKG2D antibody during co-cultivation with M-DC8$^+$ cells (Fig. 6C).

Discussion

In the present study, we evaluated specific T cell activation after antigen targeting to M-DC8$^+$ cells, which have been recently identified as a new sub-population of DC in human blood (12).

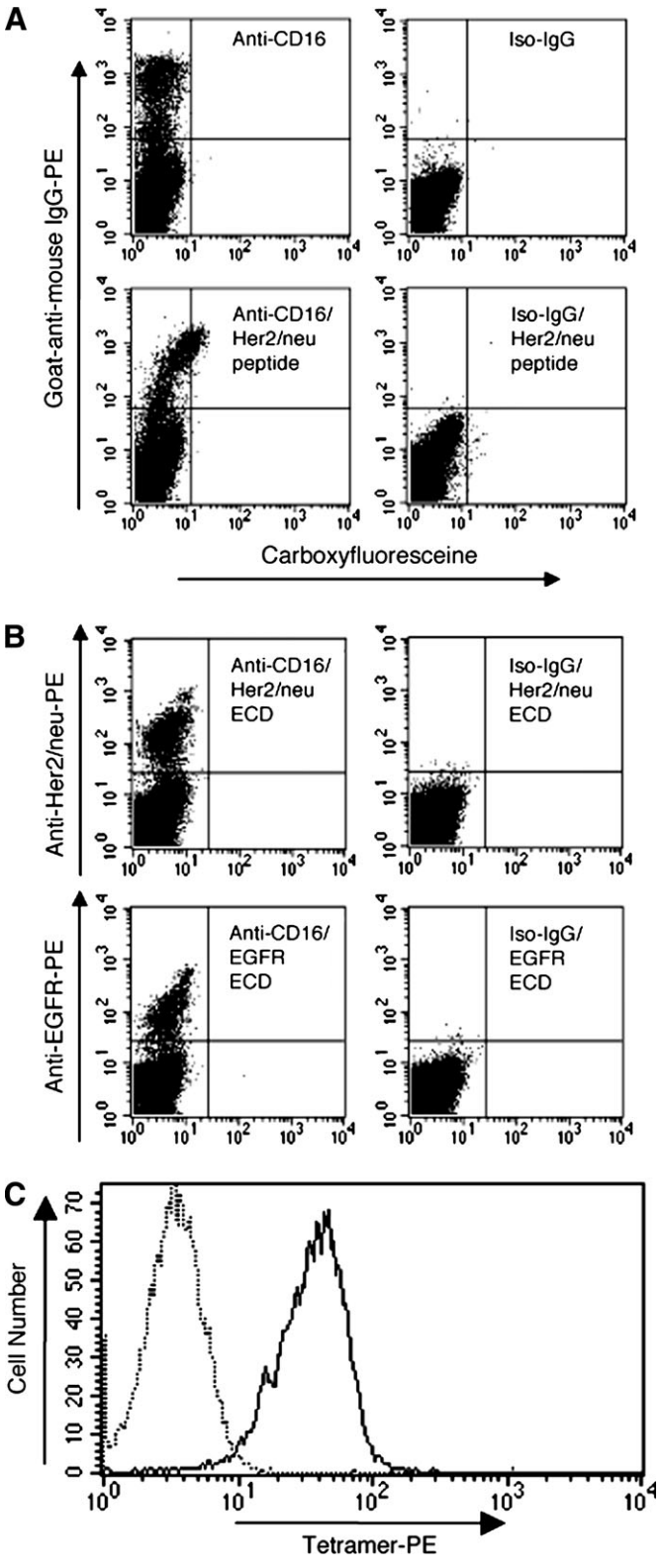

Fig. 5. Binding of anti-CD16/peptide and anti-CD16/protein conjugates to the NK cell fraction of human PBMC. (A) Cells were incubated with anti-CD16–Her2/neu peptide conjugates, an isotype-matched control conjugate of irrelevant antibody specificity (Iso-IgG/Her2/neu peptide) or complete antibody followed by staining with a PE-conjugated anti-mouse antibody for detection and flow cytometric analysis. Dead cells were excluded by PI staining. The anti-CD16 conjugate but not the Iso-IgG conjugate showed binding to M-DC8 cells. (B) Binding of antibody/Her2/neu and antibody/EGFR to human PBMC was detected by PE-conjugated antibodies directed against the antigen. Otherwise, experiments were performed as in (A). Binding activity of antibodies in the conjugates was preserved and no unspecific binding was detectable. (C) Specificity of CD8-positive T cell clone. T cells were stained with PE-conjugated Her2/neu peptide/MHC tetramer (solid line) followed by flow cytometric analysis. Staining with MHC peptide tetramer of irrelevant specificity (dotted line) was used as a control.

Human DC of the M-DC8$^+$ subtype are more frequent in human blood than those of the DC1 and DC2 subtypes, and are clearly distinguished from the latter by the expression of CD16 (23). The unique CD16 phenotype of M-DC8$^+$ cells and their abundance among the different human DC sub-populations suggested to explore FcγRIII/CD16 as a target on M-DC8$^+$ cells for antibody-based antigen delivery, although CD16 is not DC specific, but also found on a limited number of other cell types like NK cells, macrophages, neutrophils and stimulated eosinophils. We indeed could show that by the conjugation of a model antigen peptide to the anti-CD16 antibody 3G8, T cell activation induced by M-DC8$^+$ cells was enhanced by 300-fold compared with an untargeted antibody–antigen conjugate and by a factor of 500 compared with free antigen. In addition, the anti-CD16 antibody exhibited a 100-fold lower ED_{50} than the D-DC8.3 antibody that recognizes an as yet unknown structure on ~50% of M-DC8$^+$ cells. The superiority of the anti-CD16 antibody compared with D-DC8.3 seems to be mainly due to the more efficient internalization of FcγRIII/CD16 compared with the D-DC8.3 antigen, as the antibodies' binding affinities were comparable (data not shown). The efficiency of D-DC8.3 antigen conjugates might however be underestimated in our experiments because only half of the M-DC8$^+$ cells expressed high levels of the D-DC8.3 antigen.

Although the composition of the different antibody–antigen conjugates is not completely identical due to the chemical cross-linking procedure, major differences in targeting efficiency can be excluded because comparable results were obtained with different batches of antibody–antigen conjugates as well as with different model antigens (NS3 versus TT peptides). Moreover, all conjugates were normalized to statistically contain one antigen molecule per antibody.

Besides efficient antigen uptake, the general properties and the activation status of the APC are crucial for the success of vaccination strategies because antigen presentation by APC might also lead to T cell anergy (24, 25) or the induction of T cells with a regulatory phenotype (26). Interestingly, a recent study using a mouse model indicates that the immune response can be skewed from tolerance to tumor immunity by a combined application of anti-decalectin–ovalbumin peptide conjugate together with an anti-CD40 antibody to enhance the co-stimulatory capacity of DC (27), but still an APC with a high intrinsic potential to activate T cells would be favorable. As antigen uptake by receptor-mediated endocytosis or macropinocytosis is almost completely switched off in mature DC (28), only the immature DC or DC precursors can be targeted by unprocessed antigen. Cultured M-DC8$^+$ cells resemble the classical monocyte-derived immature DC with

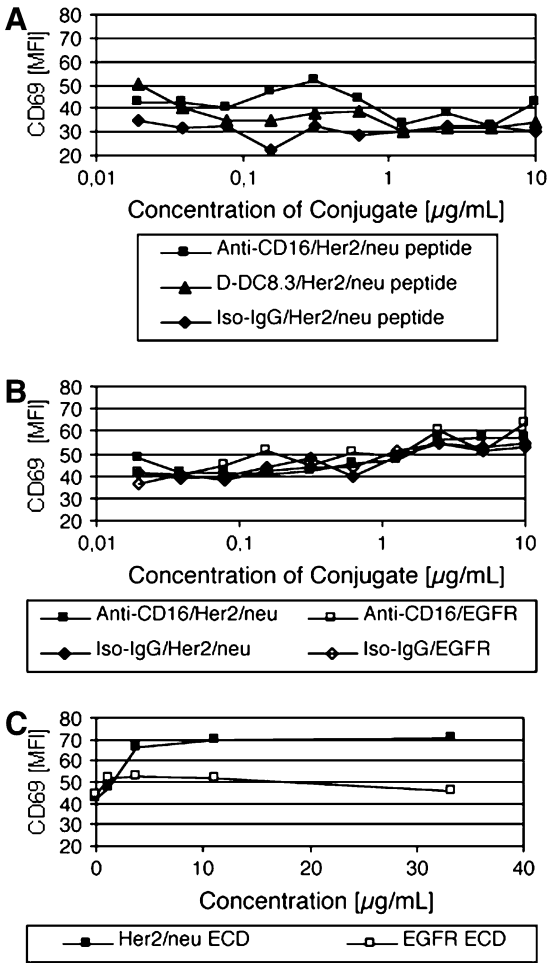

Fig. 6. Targeting of antigen to CD16 on M-DC8-positive cells does not lead to efficient cross-presentation. (A) Freshly isolated M-DC8-positive cells were incubated with titrated amounts of anti-CD16–Her2/neu peptide, D-DC8.3–Her2/neu peptide and Iso-IgG–Her2/neu peptide antibody conjugates for 14 h before washing and addition of antigen-specific cytotoxic T cells. Activation of T cells was determined by flow cytometry of CD69 expression after a 24-h cultivation period. Dead cells were excluded by PI staining and a gate was set for T cells based on the expression of CD3 (T cells) and CD11c (M-DC8-positive cells). (B) M-DC8-positive cells were pulsed with titrated amounts of conjugates consisting of anti-CD16 or isotype control antibody linked to ECD of Her2/neu. Antibody/EGFR ECD conjugates were used as control. Experiments were performed as in (A). (C) High doses of non-targeted antigen lead to cross-presentation by M-DC8-positive cells. M-DC8-positive cells were pulsed with titrated amounts of the ECD of Her2/neu or EGFR for 14 h before washing and adding Her2/neu-specific cytotoxic T cells. To increase T cell activation T cells were co-stimulated by adding 100 μg ml^{-1} of anti-NKG2D antibody during cultivation period with antigen-pulsed M-DC8-positive cells. T cell activation was determined by flow cytometric analysis as performed in (A).

regard to co-stimulatory molecules and general phenotypical appearance, and can still be targeted by anti-CD16 antibodies (13), although the expression of the FcγRIII is ~100-fold reduced compared with freshly isolated M-DC8⁺ cells. The comparison of the T cell activation capacity of freshly isolated versus cultured M-DC8⁺ DC demonstrated that the former do not only require a lower concentration of anti-CD16–antigen conjugate for induction of half-maximal T cell activation—presumably due to higher CD16 expression—but are capable of activating a higher overall percentage of T cells especially at high concentrations of antigen. The second observation proved to be independent of the way of antigen uptake and may therefore indicate that the native M-DC8⁺ cells carry a lower risk of inducing T cell anergy than immature M-DC8⁺ DC. In addition, a recent report indicates that M-DC8⁺ cells are even more potent in inducing a primary T cell proliferative response against the neo-antigen keyhole limpet hemocyanin than the classical DC1 subset (23). The general capacity of M-DC8⁺ cells to produce mainly pro-inflammatory cytokines like tumor necrosis factor-α suggests the induction of a T_h1 response upon antigen targeting (13, 23, 29), even though this has to be clarified by further experiments.

While the classical MHC class II-processing pathway for exogenous antigen is well defined and other groups have reported similar enhancement of MHC class II presentation by specific antigen targeting to different human DC-specific subsets (30–32), the requirements for efficient cross-presentation of exogenous antigen in the context of MHC class I still remain unclear. Several studies in mice indicate that FcγR can enhance cross-presentation (8, 9, 33). Because induction of cytotoxic T cells plays an important role in tumor vaccination, we analyzed the cross-presentation capability of M-DC8⁺ cells after CD16-mediated antigen uptake. Using anti-CD16–Her2/neu peptide conjugates or D-DC8.3–Her2/neu peptide conjugates analogous to those successfully used in MHC class II experiments, no activation of Her2/neu-specific cytotoxic T cells was observed as was evident from unchanged levels of CD69 expression. We could exclude that the unnatural context of flanking aa adjacent to the T cell epitope was responsible for the missing T cell activation by results with antibody conjugates containing the complete ECD of Her2/neu. We anticipate that the entire ECD preserved the natural context for T cell peptide generation through proteasomal degradation (34).

Some cytotoxic T cell clones which do not express CD28, such as the Her2/neu-specific T cell clone used here, can exhibit increased activation thresholds in that TCR–MHC class I interaction is not sufficient to induce full activation but requires additional co-stimulatory signals (35). Such a co-stimulatory signal can be provided by antibodies against NKG2D. Engagement of NKG2D has been shown to co-stimulate $CD8^+/CD28^-$ T cells (36, 37). However, the repetition of the cross-presentation experiments in the presence of anti-NKG2D antibodies did not change the outcome. Because there were low levels of unspecific T cell activation in some of the experiments, a systematic failure of T cell activation or of the respective readout system could be further excluded as a source of false-negative results. Thus, analysis of CD69 expression proved to be an appropriate and sensitive method for measuring T cell activation and antigen cross-presentation.

When M-DC8⁺ cells were incubated with unphysiologically high concentrations of untargeted Her2/neu ECD (>5-fold of maximum molar concentration of conjugates used) or with same amounts of EGFR ECD as control, we could indeed observe Her2/neu-specific T cell activation in the presence of a co-stimulating anti-NKG2D antibody. Thus, M-DC8⁺ cells proved to have the general capability of cross-presenting

exogenous antigen but it remains to be determined if cross-presentation by M-DC8$^+$ is of general relevance *in vivo*. At least, FcγRIII/CD16 does not seem to be relevant for the cross-presentation observed as antigen targeting to CD16 did not lead to efficient cross-presentation at physiological antigen levels. It is therefore likely that antigen uptake by FcγRI/CD64 is mainly responsible for FcR-mediated cross-presentation observed with immune complexes as FcγRI-targeted antigen co-localizes with MHC class I in human monocytes (38). In addition, other parameters, e.g. the degree of receptor cross-linking and activation status of the cells, will certainly influence the efficiency of cross-presentation after FcR-mediated antigen uptake. For instance, cross-presentation induced by antibody-coated tumor cells (11) or immune complexes (10) on human DC required a relatively lower dose of antigen compared with antigen targeting of the FcγRI/CD64 on monocytes with an antibody–antigen construct (39).

Early clinical trials have shown the general success of DC as natural adjuvants in cancer immunotherapy. Most trials focus on treatment of cancers like melanoma and prostate cancer where specific peptides derived from tumor-associated antigens are available (4). Vaccination of melanoma patients with DC pulsed with melanoma antigens resulted in clinical anti-tumor responses. For instance, Thurner and colleagues (40) observed regression of metastases in 6/11 patients and induction of specific CTLs in 8/11 patients after vaccination with MAGE-3A-pulsed DC. A similar approach was also successfully used for the treatment of prostate cancer patients using monocyte-derived DC pulsed with prostate specific membrane antigen peptide generating an overall response rate of 25–30% (41). In addition, several other strategies for *ex vivo* antigen loading of DC, e.g. by transfection of DC with DNA encoding tumor-relevant antigens, are currently tested in clinical trials, while all studies of antigen targeting to DC for *in vivo* vaccination purposes are still at the stage of *in vitro* or mouse models (5, 6).

In conclusion, we were able to show that antigen targeting to M-DC8$^+$ cells by anti-CD16 antibodies is an exceptionally efficient way of activating CD4$^+$ T cells. Although DC have been identified as the optimal natural adjuvants for immunization, clinical application was so far limited by the requirement of isolating autologous DC or their precursors from each individual patient. For efficient vaccination, DC were then loaded *ex vivo* with antigen and matured prior to re-injection into the patient (6). As native M-DC8$^+$ cells without further manipulations are even more potent T cell activators after CD16-mediated antigen targeting than are immature DC derived thereof by *in vitro* differentiation, antibody-based *in vivo* targeting of antigen to the surface of M-DC8$^+$ cells appears as a promising vaccination strategy, avoiding complicated individual-specific *ex vivo* steps.

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Abbreviations

aa	amino acids
APC	antigen-presenting cells
ECD	extracellular domain
ED_{50}	half maximal effective dose
EGF	Epidermal growth factor
FL	fluorescein-coupled
F/P	fluorochrome-to-protein ratio
GM-CSF	granulocyte macrophage colony-stimulating factor
NS3	non-structural protein 3
PI	propidium iodide
TT	tetanus toxoid

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