Published in final edited form as:

Vaccine. 2010 November 16; 28(49): 7764–7773. doi:10.1016/j.vaccine.2010.09.054.

Dendritic cells transfected with Her2 antigen-encoding RNA replicons cross-prime CD8 T cells and protect mice against tumor challenge

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Abstract

Antigen-specific T cells can be induced by direct priming and cross-priming. To investigate cross-priming as a vaccination approach dendritic cells were transfected with cytopathogenic viral RNA-replicons that expressed domains of the tumor-associated Her2-antigen and injected into MHC-discordant mice that did not allow direct priming. Upon tumor challenge 75% of the vaccinated, but none of the mock-vaccinated mice remained tumor-free. The anti-tumor effect required T cells and correlated with the vigor of the cross-primed CD8 T cell response. Her2-specific antibodies were not detected. This study highlights the potential of T cell cross-priming in cancer immunotherapy.

Keywords

Cross-priming; Her2; RNA replicon

1. Introduction

Tumor associated antigens (TAAs) are primarily expressed by tumor cells and frequently involved in tumorigenesis [1]. Immunotherapy as an alternative type of cancer treatment aims at stimulating the immune system to selectively react against neoplastic cells by breaking the tolerance to TAA [2]. A particularly promising approach takes advantage of the unique properties of dendritic cells (DCs) to induce and regulate immune responses. Subsequent to antigen acquisition, DCs mature and migrate to secondary lymphoid organs where they present immunogenic peptides in the context of major histocompatibility complex (MHC) molecules to T cells and deliver critical co-stimulatory signals [3]. Following the first clinical study that employed autologous, peptide-pulsed DCs for vaccination of patients with B cell lymphoma [4] multiple trials were conducted, which showed DC vaccination to be safe, well tolerated and immunogenic [5].

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The immunogenicity of DC-presented foreign antigens depends on the activation/maturation status of the cells, on the efficiency of antigen processing, and on a sustained presentation of high numbers of immunogenic peptides on the MHC [6,7]. Thus, a critical determinant of the immunogenicity of DC-based vaccines is the “loading” of vaccine DCs with antigen. For example, this is achieved by pulsing DCs with recombinant tumor proteins or peptides, or by transfecting DCs with antigen-encoding nucleic acids [5]. Another important determinant of the immunogenicity of DC-based vaccines is the transfer of cellular material from vaccine DCs to endogenous antigen presenting cells (APCs) [6]. The latter comprise macrophages and endogenous DCs [8], which internalize proteins, cellular fragments and apoptotic cells [9] and reprocess antigens in the endosomal compartment or cytosol for presentation on their own MHC molecules [8,10]. Vaccination with antigen-expressing DCs may induce rapid, effective and maintained T cell responses against viral or tumor antigens that are otherwise not accessible to endogenous APCs. This immunological pathway is termed “cross-priming” and may be particularly useful in vaccination approaches [11,12].

In a recent study, we used the cross-priming pathway to induce a protective T cell response against hepatitis C virus [13]. For this purpose, vaccine DCs were transfected with self-replicating viral RNAs (“RNA replicons”) of bovine viral diarrhea virus (BVDV), in which the genetic units coding for the virus structural proteins were replaced by a heterologous open reading frame coding for an antigen of choice (Fig. 1A). Such “bi-cistronic” BVDV replicons turned out to be advantageous for vaccination because they express high amounts of the antigen in the cytoplasm of the transfected cells and do not form infectious virus particles. An additional advantage is the cytopathogenicity of BVDV replicons that express the viral NS3 protein resulting in apoptosis 24–48 h after transfection [14,15]. This time-delayed apoptosis of the replicon-transfected vaccine DCs was shown to be crucial for efficient cross-priming of an antigen-specific T cell response [13].

The aim of the current study was to employ the DC/BVDV replicon system to vaccinate against neoplastic cells that over-express Her2 as a model TAA. Her2 (ErbB2; neu) is overexpressed by breast tumors, gastric carcinomas, lung tumors and ovarian cancer [16]. In an attempt to enhance the vaccination process, the DC/replicon system was used to co-deliver the cytokine IL-12, which plays a key role in the activation of T cells. Our results demonstrate that the DC/BVDV replicon system can be used as a valuable tool in vaccination approaches targeting tumor cells as well as a delivery system of immuno-stimulatory molecules. Most interestingly, induction of T cell responses via cross-priming was shown to be sufficient to induce a potent, preventive anti-tumor response in the absence of direct priming of T cells, and in the absence of antibodies.

2. Materials and methods

2.1. Cell lines

The dendritic cell line DC2.4 (haplotype: H-2^b) was kindly provided by Dr. K.L. Rock (Dana Farber Cancer Institute, Boston, MA) [17] and cultured in RPMI 1640 containing 10% heat-inactivated bovine calf serum (FBS), 2 mM L-glutamine, 100 µM nonessential amino acids, 100 U/ml penicillin/streptomycin (all from Cellgro, Manassas, VA) (complete medium) and 50 µM 2-mercaptoethanol (Gibco BRL, Grand Island, NY). Sublines of highly transfectable cells were established by limiting dilution cloning. The rat Her2-expressing mammary tumor cell line NT-2 (haplo-type: H-2^d) [18] was kindly provided by Dr. E.M. Jaffee (Johns Hopkins Medicine, Baltimore, MD) and grown in complete medium supplemented with an additional 10% FBS, 10 mM Hepes, 1% Napyruvate, and 100 mU/ml human insulin (Eli Lilly, Indianapolis, IN). MDBK cells (ATCC, Rockville, MD) were cultured in DMEM (Lonza, Cologne, Germany) with 5% heat-inactivated bovine fetal calf serum (FCS), 100 µU/ml penicillin/streptomycin, 1% hypoxanthine and 1% β-biotin

(AppliChem, Darmstadt, Germany). Rat Her2-expressing 3T3/neu cells and wildtype NIH/3T3 cells (haplotype: H-2^q) (ATCC) were cultured in RPMI 1640 supplemented with 10% FCS and 100 U/ml penicillin/streptomycin with and without 300 nM methotrexate (Sigma–Aldrich, St. Louis, MO), respectively.

2.2. Generation of recombinant replicons

cDNA templates of bi-cistronic replicons encoding either the rat Her2 extracellular domain (ECD), the rat Her2 middle fragment (MF) or the mouse interleukin 12 (IL-12) gene were generated in four steps. First, FLAG epitope-encoding oligonucleotides that contained several restriction sites were designed for each replicon insert and cloned into pBluescript KS+ (Stratagene, La Jolla, CA) using the restriction enzymes XhoI and NotI (New England Biolabs, Ipswich, MA, or Fermentas, St. Leon-Rot, Germany). Second, the template vectors for *hHer2* (pLNCX-Her2; kindly provided by Dr. L. Weiner, Fox Chase Cancer Center, Philadelphia, PA) and *mIL-12* (pORF-mIL-12, InvivoGen, San Diego, CA) were digested with AatII and NdeI to generate the Her2 ECD fragment, or with BglII and NcoI to generate the Her2 MF fragment or with AvrII and NcoI to generate the IL-12 gene. The resulting Her2 gene fragments and the IL-12 gene, respectively, were cloned into the plasmids from the first cloning step downstream of the FLAG coding sequence. Third, we utilized a pSP64 derivative that contained the SP6 promoter, the BVDV 5' UTR and the N^{PRO} gene upstream of the hepatitis C virus NS3 gene (construction details provided on request). FspI and SalI were applied to exchange most of the HCV NS3 gene by the sequences encoding FLAG and the heterologous Her2 ECD, Her2 MF and IL-12. Fourth, the resulting plasmid constructs were cut with NheI and SalI and the NheI/SalI fragments encoding the SP6 promoter, the BVDV 5' UTR, the N^{PRO} sequence, and FlagHer2ECD, Flag Her2MF or Flag IL-12 inserted into the Bi-ubi-NS3-NS5B cDNA platform cut with NheI and XhoI [13].

2.3. In vitro transcription of RNA

DNA templates of Her2 and IL-12 replicons were linearized with SrfI and SmaI (Stratagene, La Jolla, CA), respectively. After purification with MiniElute Reaction Cleanup Kit (Qiagen Inc., Valencia, CA), linearized plasmids were in vitro transcribed with SP6 RNA polymerase (Roche Diagnostics, Indianapolis, IN). The DNA template was removed by digestion with RNase-free DNase I (Roche Diagnostics, Indianapolis, IN) and the RNA was purified with the Rneasy Mini Kit (Qiagen Inc., Valencia, CA). RNA concentration and integrity were determined by UV spectrophotometry (OD 260 nm) and agarose gel electrophoresis.

2.4. In vitro translation

RNA template (1 µg) was incubated with 30% (v/v) S10 extract prepared from Huh7 cells [19], 1 mM ATP (Roche Applied Sciences, Mannheim, Germany), 0.2 mM GTP (Roche Applied Sciences), 120 mM potassium acetate (Sigma–Aldrich, Deisenhofen, Germany), 2.6 mM magnesium acetate, 30 mM Hepes buffer, 3 mM DTT, 50 mM creatine phosphate (Roche Applied Sciences), 0.8 µg/ml creatine kinase (Roche Applied Sciences), 0.8 U/µl RNasin (Promega, Mannheim, Germany) and 10–15 µCi [³⁵S] methionine (GE Healthcare, Munich, Germany) for 4 h in a volume of 25 µl at 30 °C. Proteins were separated on a 12% SDS-PAGE and the radioactively labeled translation products were analyzed by autoradiography using a phosphoimager (GE Healthcare, Munich, Germany).

2.5. RNA transfection

DC2.4 and MDBK cells were transfected with replicon RNA using the Gene Pulser II apparatus (BioRad, Hercules, CA). Cells were split 16–24 h prior to transfection, washed with PBS and re-suspended at 10⁶/ml PBS. DC2.4 cells were electroporated with 5 µg RNA

at 300 V, 750 μ F and 400 Ω in a 4-mm cuvette. MDBK cells were electroporated with 3 μ g RNA at 180 V, 950 μ F with resistance set to ∞ in a 2-mm cuvette. Transfection efficiencies were around 30% for DC2.4 cells and 80% for MDBK cells as determined by BVDV NS3 expression analyzed by fluorescence microscopy or by flow cytometry.

2.6. Analysis of replicon-induced cell death

DC2.4 cells were collected at 15 h, 35 h and 51 h post transfection with the replicon *rHer2MF* by combining the cell culture supernatant and the trypsinized cells. After staining with LIVE/DEAD® Fixable Violet Dead Cell Stain (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, the cells were permeabilized with BD Cytotfix/Cytoperm™ (BD Biosciences, San Jose, CA, USA) and indirectly stained for NS3 (1:50 dilution of anti-NS3 hybridoma culture supernatant [20] kindly provided by Dr. N. Tautz, University of Lübeck, Germany; secondary antibody: PE-labeled rat-anti-mouse IgG F(ab)₂ fragment, Invitrogen). Cells were analyzed using an LSRII flow cytometer (BD Biosciences), FACS Diva Version 5.0 (BD Biosciences) and FlowJo Version 8.3 (Tree Star, Ashland, OR) software.

2.7. Analysis of protein expression

2.7.1. Western blot—MDBK cells were harvested 24 h after transfection, washed twice in ice-cold PBS, and incubated for 30 min in 1 ml cell lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor mix [Roche Diagnostics, Indianapolis, IN]). Cell lysate proteins were separated on a 10% SDS PAGE and transferred to a nitrocellulose membrane by semi-dry-blotting. The membrane was incubated with 1:1000 diluted mouse-anti-Flag M2 (Sigma–Aldrich GmbH, Deisenhofen, Germany) and subsequently with 1:7500 diluted secondary, horseradish peroxidase coupled anti-mouse-IgG (Amersham Biosciences Europe GmbH, Freiburg, Germany). The membrane was treated with ECL solution (Pierce, Rockford, IL) for 5 min and exposed to an X-ray film.

2.7.2. EIA—DC2.4 cells were transfected with Repl-*mIL12* or Repl-*rHer2MF* in triplicate cultures and the supernatants were collected 14 h, 24 h and 48 h after transfection and immediately frozen at -20°C . After thawing the samples, cells and cell debris were pelleted by centrifugation at 13,000 g for 10 min at 4°C . The supernatant was diluted four-fold and subjected to an ELISA specific for the bio-active form of murine IL-12 (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

2.8. Mouse vaccination and tumor challenge

All mouse experiments were performed with FVB/N mice under a protocol approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases. Eight to ten week-old FVB/N mice (haplotype: H2^d) (Jackson Laboratory, Bar Harbor, ME) were vaccinated subcutaneously at the base of the tail with 10^6 replicon-transfected DC2.4 cells in 200 μ l PBS and boosted 3–5 weeks later. Transfection was performed 14 h prior to vaccination. In some experiments, mice were vaccinated with two DC2.4 preparations, each transfected with a different replicon. Control mice were immunized with mock-transfected DC2.4 cells (electroporated in the absence of RNA). Two weeks after the second vaccination, the mice were challenged by subcutaneous injection of 10^6 NT-2 tumor cells into the right flank. Tumor growth was monitored thrice weekly by measuring two dimensions with a caliper (largest dimension: *a*; smallest dimension: *b*), and tumor volume (*V*) was calculated with the following formula: $V = (a \times b^2)/2$. A minimum of eight mice were tested for each vaccination condition.

2.9. Depletion of T cell subsets

Mice were intraperitoneally injected with either 1 mg of purified anti-CD4-antibody (Harlan laboratories, Indianapolis; clone GK1.5) or 1 mg of purified anti-CD8-antibody (Harlan laboratories; clone 2.43) on days -7, -2, 5, 12 and 19 relative to the tumor challenge. To confirm T cell depletion, splenocytes were stained with ethidium monoazide (Sigma–Aldrich), CD3-PacificBlue (500A2), B220-PE-Cy5 (RA3-6B2), CD8-PE-Cy7 (Ly-2) and CD4-APC-Cy7 (L3T4) (all antibodies were obtained from BD Biosciences) and analyzed with the LSRII flow cytometer (BD Biosciences) prior to and on day 22 post tumor challenge, respectively. Depletion efficiency was determined by dividing the frequency of the targeted cells in depleted and undepleted mice.

2.10. Detection of Her2-specific T cells

CD8 T cells were isolated with magnetic beads (Miltenyi, Bergisch Gladbach, Germany) from spleens of vaccinated mice 3 weeks after tumor challenge (manufacturer's protocol). Serial dilutions of CD8 T cells were resuspended in serum-free HL-1 medium (BioWhittaker, Walkersville, MD), supplemented with 2 mM L-glutamine and added to anti-IFN- γ (BD Biosciences, clone R4-6A2) coated wells of MultiScreenHTS plates (Millipore, Billerica, MA, USA). The T cells were then incubated with 5×10^5 irradiated (10,000 rad) 3T3 cells and either (i) pools of 20mer peptides (Mimotopes, Clayton, Australia) that overlapped by 10 amino acids and covered amino acids 385–625 of the rHer2 protein (1 μ g/ml of each peptide), or (ii) PBS/DMSO without peptides. After 40 h of incubation, the plates were washed three times with PBS and four times with PBS/Tween (1:1000), followed by an overnight incubation with a biotin-labeled IFN- γ antibody (BD Biosciences, clone XMG1.2), a 1 h incubation with streptavidin diluted 1:2000 in PBS/Tween and development of the plate with the AP conjugate substrate kit (Biorad). Spots were counted with an AID EliSpot Reader Version 3.5 (Autoimmun Diagnostika GmbH, Straßberg, Germany). The number of specific spots was calculated by subtracting the number of spots in the negative control wells from the number of spots in Her2-peptide stimulated wells.

2.11. Detection of Her-2-specific antibodies

The presence of Her2-specific antibodies in vaccinated mice was analyzed using a modification of a protocol by Reilly et al. [18]. Briefly, blood was collected by retroorbital bleeding ten days after the second vaccination. 2×10^5 3T3 or 3T3/neu cells, respectively, were pre-incubated for 10 min with Fc γ -blocking antibody (clone 2.4G2, BD Biosciences) and then incubated with undiluted plasma at 4 °C for 30 min. Bound antibodies were detected by secondary staining with a PE-labeled anti-mouse-IgG (Invitrogen, Carlsbad, CA) followed by flow cytometry. Plasma from mock-vaccinated mice collected prior to tumor challenge served as a negative control, and plasma collected three weeks after tumor challenge was used as positive control.

2.12. Statistical analysis

Statistical analysis was performed using two-tailed tests and Graph Pad Prism software (GraphPad Software, Inc., LaJolla, CA, USA). The Mann–Whitney *U* test was used to compare tumor sizes among groups of mice. Frequencies of tumor-free mice or mice with a significant CD8 T cell response were compared between groups using the Chi-square test. Linear regression was used to examine correlations between the tumor size and the CD8 T cell response. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Generation of cytokine and antigen expressing RNA replicons

The initial task of this study was the construction of cDNAs that allowed the transcription of bi-cistronic BVDV replicon RNAs encoding IL-12 and immunogenic domains of Her2. While the procedures for the generation of the cDNA platforms were the same as described by Racanelli et al. [13] (see Section 2 for details), the heterologous ORFs were constructed in such a way that a fusion protein comprising (N- to C-terminus) the pestiviral autoprotease N^{PRO} and the heterologous protein was generated. Accordingly, the N-termini of the expressed proteins were generated by autoproteolytic cleavage of N^{PRO} [21]. Moreover, each protein contained a FLAG epitope for immunodetection (Fig. 1A).

For the expression of functional IL-12, the entire mouse IL-12 gene (*mIL-12*, ca. 1.7 kb) encoding the polypeptide-linked p35- and p40 subunits was cloned into the cDNA platform. Concerning Her2, we used the rat Her2 sequence (*rHer2*), because *rHer2*-expressing NT-2 tumor cells were available to challenge the vaccinated mice [18]. Since replicons encoding the entire *rHer2* ORF (ca. 3.4 kb) replicated only at low levels (data not shown), we decided to express only those parts of the protein that encoded MHC class I epitopes known to be recognized by FVB/N mice (Fig. 1B). Thus, gene fragments encoding amino acids 175–627 of Her2 (corresponding largely to the protein's ectodomain, ECD) and amino acids 373–1005 (corresponding to the protein's middle fragment, MF) were inserted into the cDNA. The generated replicon RNAs were referred to as Repl-*mIL12*, Repl-*rHerECD* and Repl-*rHerMF*, respectively.

3.2. Assessing the recombinant RNA replicons as cytokine and antigen delivery systems

Following standard procedures, all replicon constructs were generated by in vitro transcription. Pilot experiments demonstrated that the constructs replicated at comparable levels (data not shown) and in multiple transfected host cells, including the murine DC2.4 cell line [17] that was used for vaccination in this study (Fig. 2).

To evaluate the cytopathogenic properties of the viral RNAs, the number of transfected and dying cells was determined at different time points after transfection of DC2.4 cells. The percentage of replicon-transfected cells was quantified by immuno-staining of the BVDV NS3 protein (note that only cells containing replicating viral RNA are detectable by this procedure [14]) and the percentage of dying cells was determined by flow cytometry. 15 h after transfection, which corresponds to the time point when the replicon-transfected DCs were used for vaccination (see below), only low levels NS3 expression were detected (Fig. 2A). About 10% of the total DC2.4 population consisted of dead or dying cells at this time point, which was mostly attributed to the transfection procedure itself rather than to a replicon-specific effect. Conversely, 35 h after transfection, intracellular NS3 expression was detected in 25% of the RNA replicon-transfected cells. Forty percent of this population of transfected cells were dying or dead (Fig. 2A). Finally, 51 h after transfection, only a few vital cells remained detectable. These data revealed a clear correlation between viral RNA replication (as measured by the expression of the BVDV NS3 protein) and replicon-induced cell death.

Next, we evaluated the generation of the heterologous, replicon-encoded proteins. As shown in Fig. 2B, IL-12 was detectable by western blot in cytoplasmic extracts of Repl-*mIL12* transfected MDBK cells 24 h after transfection. To verify that functional IL-12 was produced and released from transfected DCs, we collected the supernatants of Repl-*mIL12* transfected DC2.4 and of Repl-*rHerMF* transfected DC2.4 (used here as negative control) 14 h, 24 h and 48 h post transfection and subjected them to an EIA specific for the bioactive form of the cytokine (Fig. 2B). IL-12 was detectable in the supernatant of Repl-*mIL12*-

transfected, but not in the supernatant of Repl-*rHerMF*-transfected DCs as early as 14 h after transfection, i.e., at a time point at which a significant cytopathic effect had not yet occurred. Fourteen to 24 h after transfection, the cumulative amount of produced IL-12 had increased approximately 6-fold (Fig. 2B), which was consistent with increased production of BVDV NS3 protein in this time frame (Fig. 2A). Collectively, these results demonstrated that IL-12 was produced in its bioactive form and released by Repl-*mIL12*-transfected DCs. The level of protein production was similar to that observed with other bi-cistronic replicon constructs [15], i.e., ca. 200 ng of secreted protein per 10^7 cells.

In contrast to IL-12, which was expressed as intact protein with a relatively long half-life time of 30 h [22], the Her2-fragments ECD and MF were undetectable in western blots of lysates of DCs transfected with Repl-*rHerECD* and Repl-*rHerMF*, respectively (data not shown). Assuming that the short half-life time of the Her2 protein fragments hampered their detection in transfected cells, we decided to verify the overall protein expression profiles of Repl-*rHerECD* and Repl-*rHerMF* by in vitro translation side-by-side with the BVDV replicon DI9c, which encodes solely N^{pro} and NS3-NS5B (Fig. 1A). For this purpose, we employed a protocol that was earlier shown to support efficient translation as well as correct maturation of the DI9c-encoded nonstructural polyprotein in vitro [19] (see Section 2). Indeed, the Repl-*rHerECD* and Repl-*rHerMF* RNAs expressed the correctly processed *rHerECD* (50 kDa) and *rHerMF* (68 kDa) proteins and the corresponding N^{pro}*rHerECD* (69 kDa) and N^{pro}*rHerMF* (87 kDa) precursors in addition to the BVDV proteins (Fig. 2C).

In conclusion, all replicon constructs displayed the expected properties: the RNAs replicated efficiently in transfected DC2.4 cells, caused a cytopathogenic effect within the expected time frame and expressed the encoded heterologous proteins of interest.

3.3. Vaccination with Her2 replicon-transfected DCs protects mice against tumor challenge

To evaluate replicon-transfected DC2.4 cells as an anti-tumor vaccine we followed a vaccination regimen in which FVB/N mice were vaccinated and boosted 3–5 weeks later. For this purpose, mice were subcutaneously vaccinated with DC2.4 cells that had been transfected with the individual replicons Repl-*rHerECD*, Repl-*rHerMF* or Repl-*mIL12* (referred to as ECD, MF and IL-12 groups) or with combinations of *rHer2* and Repl-*mIL12* replicons (ECD/IL-12 and MF/IL-12 groups). Mice vaccinated with mock-transfected DC2.4 cells served as negative controls. Two weeks after the booster, the vaccinated mice were challenged by a subcutaneous injection of *rHer2*-expressing NT-2 tumor cells (see Section 2). Importantly, due to the disparate MHC haplotypes of the vaccine DC2.4 cells (H-2^b) and the FVB/N mice (H-2^q) direct priming of T cells was not feasible. Thus, induction of Her2-specific T cells was only possible by cross-priming (see also below).

As shown in Fig. 3, DC2.4 cells transfected with Her2-expressing replicons mediated a significant anti-tumor effect. That is, approximately 33% of the mice in the ECD group and approximately 50% of the mice in the MF group remained tumor-free by the end of the experiments, which was three weeks after challenge with *rHer2*-expressing NT-2 tumor cells. On the contrary, none of the mock-vaccinated control mice remained tumor-free (Fig. 3A). Interestingly, co-administration of DC2.4 cells transfected with Repl-*mIL12* and DC2.4 cells that were transfected with either of the *rHer2* replicons showed a trend towards better protection [75% (ECD + IL-12) vs. 33% (ECD) of mice remained tumor-free, 71% (MF + IL-12) vs. 50% (MF) of mice remained tumor-free]. In contrast, administration of DC2.4 cells transfected solely with Repl-*mIL12* protected only 20% of the mice. In addition, mice that were vaccinated with either Repl-*rHerECD*-transfected or Repl-*rHerMF*-transfected DCs developed significantly smaller tumors than the mock-vaccinated mice (Fig. 3B, C), while no significant difference was observed in mice that were vaccinated solely with the IL-12 encoding Repl-*mIL12*.

Taken together, Her2 replicon-transfected DCs mediated a significant anti-tumor effect in a preventive vaccination approach. Both Her2-replicons, irrespective of whether the ECD or MF portions of the protein were expressed, were equally efficient. Co-delivery of IL-12 did not significantly increase the vaccination effect though a trend towards complete protection against tumor growth was observed.

3.4. T cells are essential for the DC/replicon-mediated anti-tumor response

The above experiments demonstrated a significant anti-tumor effect of Her2 replicon-transfected DC2.4 cells and IL-12 did not significantly increase this effect. To identify the protective T cell subset, groups of mice vaccinated with Repl-*h*HerMF and Repl-*m*IL12-transfected DC2.4 cells were depleted of CD4 or CD8 T cells. Greater than 99.9% of the CD4 T cells and greater than 99% of the CD8 T cells were depleted throughout the experiment, i.e., up to three weeks after tumor challenge (Fig. 4A).

Consistent with the preceding experiments (Fig. 3), the majority (4/6) of the undepleted, DC/Repl-*h*HerMF and Repl-*m*IL12-vaccinated mice were fully protected against tumor growth, and the remaining (2/6) mice developed only small tumors upon challenge (Fig. 4B and C). In contrast, all CD4- and CD8-depleted mice developed tumors, and these tumors were considerably larger than those of mock-vaccinated mice (Fig. 4C). Albeit not significant, we observed a trend towards greater tumor growth in CD8-depleted mice than in CD4-depleted mice. Collectively, these data demonstrated that both, CD4 and CD8 T cells, contributed to the vaccine-induced immune response.

3.5. Inverse correlation of CD8 T cell response and tumor size

The finding that CD8 T cell depletion diminished the anti-tumor effect was consistent with the generally accepted concept that the induction of CD8 T cells is crucial for cancer immunotherapy. To verify that protection was indeed mediated by Her2-specific CD8 T cells, we performed IFN- γ ELISpot analyses 3 weeks after tumor challenge. As shown in Fig. 5A and B, vaccination with DC2.4 cells that had been transfected with the Her2 replicons or with Her2 replicons plus Repl-*m*IL12 indeed induced a substantial number of Her-2-specific, IFN- γ -producing T cells. In contrast, mice that were vaccinated with mock-transfected or with Repl-*m*IL12-transfected DC2.4 cells displayed a weak CD8 T cell response (Fig. 5C).

Based on the data obtained with the mock and IL-12 vaccination groups, we defined “strong” as a CD8 T cell response of greater than 100 SFU per 1.25×10^5 CD8 T cells (Fig. 5A–C, red lines and red symbols) and “weak” accordingly as a response below this arbitrary threshold (Fig. 5A–C, black lines and black symbols). Following this definition, only 1 out of 12 mice (8%) in the mock and IL-12 vaccination groups developed Her2-specific CD8 T cells. In contrast, significantly more mice in the groups vaccinated with Her2-encoding replicons showed a strong CD8 T cell response (10 out of 23, 43%; $p = 0.0335$) (Fig. 5A and B). In particular, the combination of Repl-*h*HerMF-transfected and Repl-*m*IL12-transfected DC2.4 cells induced the strongest Her2-specific CD8 T cell response: 4/6 mice (67%) in this group showed a CD8 T cell response above the cut-off, as compared to 2/8 mice (25%) in the ECD group, 2/4 mice (50%) in the ECD + IL-12 group and 2/5 mice (40%) in the MF group (Fig. 5B). Importantly, mice with strong CD8 T cell responses displayed the smallest tumors (Fig. 5A–C, second and fourth graphs from the left) and an inverse correlation between strength of the CD8 T cell response and tumor sizes was shown for all mice ($p = 0.0016$, $r^2 = 0.26$; Fig. 5D).

In sum, these findings showed that vaccination with replicon-transfected DCs induced CD8 T cells that specifically targeted the encoded Her2 TAA. Since the injected DC2.4 cells and

FVB/N mice differed in their MHC class I haplotypes, the Her2-specific CD8 T cells were exclusively induced by cross-priming.

3.6. Antibodies are not significantly involved in the DC/replicon induced anti-tumor response

The final experiment of this study was designed to understand the role of the humoral immune system in the observed anti-tumor response. For this purpose, we analyzed the plasma of vaccinated mice for Her2-specific antibodies 10 days after vaccination. Plasma samples were incubated with *r*Her2-expressing 3T3 cells (3T3/neu) to allow the binding of antibodies to the Her2 antigen on the cell surface. Bound Her2-specific antibodies were then detected with a fluorophore-conjugated secondary anti-mouse IgG by flow cytometry. Plasma from mice immunized with mock-transfected DCs prior to tumor challenge served as a negative control and plasma from mock-vaccinated mice after tumor challenge as a positive control, respectively. Her2-negative 3T3 cells served as an additional control. As shown in Fig. 6A, Her2-tumors induced specific antibodies (Fig. 6A) but vaccination with Her2 replicon-transfected DCs did not (Fig. 6B, C). These data demonstrate that Her2-specific antibodies were not induced by DC/replicon vaccination and hence did not contribute to the observed anti-tumor effect.

4. Discussion

Multiple phase I and II clinical trials proved DC-based immunotherapies to be safer and less toxic than conventional cancer therapies. However, to date, the response to DC-based cancer immunotherapies is frequently unsatisfying as exemplified by the complete failure of a phase III trial in melanoma patients [23]. Accordingly, various parameters such as the DC maturation status, the route of administration and, most prominently, the mode of antigen-delivery require further improvement [24]. Many studies to evaluate antigen-delivery strategies were performed in mice, several of them using Her2 as a model TAA. In preventive vaccination models, a delayed tumor onset was achieved with DCs loaded with a heteroclitic variant of an Her2 peptide [25]. In addition, DCs loaded with virus-like particles of murine polyoma virus that contained a Her2 subdomain fusion protein protected against challenge with Her2-expressing cells [26]. Finally, DCs that were transduced with a replication deficient adenovirus encoding the Her2 extracellular and transmembrane domains not only protected vaccinated mice against challenge with Her2-expressing TUBO cells but also eliminated even established tumors and metastases [27]. While the latter approach was most promising, concerns regarding safety and pre-existing immunity remain about vaccines that apply human pathogenic viruses as delivery systems.

In the present study, we used cytopathogenic BVDV replicons to load vaccine DCs with the Her2 TAA. Thus, a strategy that we originally developed to induce an anti-viral immune response [13] was translated to anti-tumor vaccination and shown to induce an antigen-specific T cell response and to mediate a preventive anti-tumor effect (Figs. 3–5). While the use of syngeneic DC would be more suitable for therapeutic applications in humans, we used allogeneic DC in this study for the following reason: the use of replicon-transfected DC2.4 cells of the H-2^b MHC haplotype to vaccinate mice of the H-2^d haplotype, allowed us to obtain the most important result of this study, the demonstration that cross-priming of T cells is sufficient to mediate a significant preventive anti-tumor response. While cross-priming was already shown to be relevant for the induction of immune responses against viruses such as herpes simplex virus 1 [28] and modified vaccinia virus Ankara [12], cytopathic BVDV replicons turned out to be particularly suitable to induce efficient cross-priming due to the time-delayed cytopathic effect of the replicating RNA. That is, apoptosis of the transfected DCs occurs 24–48 h after transfection (Fig. 2), which leaves sufficient time for the migration of the vaccine DCs to the secondary lymphoid organs, the MHC-

restricted presentation of antigen/antigenic peptides on the cell surface, and, perhaps, the expression of danger signals that may act as adjuvants, for example via the TLR pathway [29,30] (see also below).

Several additional facts support our conclusion that BVDV replicons are useful vaccination tools. In contrast to viral vectors and RNA replicons of Semliki Forest virus [31], Kunjin virus [32], Venezuelan equine encephalitis virus [33] or Sindbis virus [34] that were utilized in previous studies, the BVDV replicons derive from an animal virus that does not infect humans. Hence, many safety issues do not apply to BVDV, and pre-existing immunity does not exist. The use of replicons to load DCs combines the advantages of using antigen-encoding mRNA with that of using viral vectors. RNA replicons are not infectious, and gene expression does not involve integration into the host genome. Replication of the replicon RNA mimics viral infections, and mediates adjuvant effects. In particular, double-strand RNA (dsRNA) replication intermediates of (+)-strand RNA viruses were recently shown to activate the Toll-like receptor TLR-3 pathway [35]. Moreover, infections with cytopathogenic viruses were found to stimulate TLR-2, -4 and -7 expression [36]. TLR signaling induces the differentiation and maturation of DCs and enhances antigen presentation [37]. Finally, the cytopathogenic form of BVDV is known to induce the expression of type I interferons (IFN- α/β) [37], which have been described to enhance cross-priming [38].

Preventive vaccination with the DC/BVDV replicon system resulted in the suppression of tumor growth in a subset of the mice complete protection against tumor development. In a depletion experiment (Fig. 4), we demonstrated indispensable roles of CD8 and CD4 T cells for this immune response. These data are consistent with previous Her2 vaccination studies that demonstrated the requirement of CD8 T cell activation for a significant anti-tumor effect [39–41]. The activation of CD8 T cells is crucial for cancer immunotherapy as endogenous tumor antigens are generally presented via MHC class I. Accordingly, tumor infiltrating CD8 T cells have been associated with a better prognosis [42], and adoptive transfer of CD8 T cells is highly effective against solid tumors, e.g. in melanoma patients [43].

CD4 T cells contribute to the antitumor effect as shown in the depletion experiments even though there was no antibody production. Since CD4 T cell depletion was performed four weeks after the first vaccination and one week after the booster vaccination it is possible that CD4 T cells facilitate the priming of CD8 cells [44], or that they secrete immunostimulatory cytokines and activate innate immune cells. CD4 T cells may also exert direct effector functions and kill tumor cells expressing MHCII molecules [45] and facilitate the recruitment of CD8 T cells to tissues that harbor the antigen-expressing cells [46].

We attempted to further improve the priming of anti-tumor T cells by co-delivery of IL-12. IL-12 was already tested in several immunotherapy studies [47], and its paracrine application was found to be safe and efficient. However, while we observed a trend towards better protection against tumor growth, this effect was not significant (Figs. 3–5). While earlier reports showed a clear beneficial effect of IL-12 in vaccination approaches with TAA-encoding DCs [39] the adjuvant effects of the cytopathic BVDV replicon may have rendered this unnecessary in our system.

Likewise, the humoral immune response was not required in our vaccination model (Fig. 6). While antibody-independent immune responses against tumors were previously observed [40,41,48], several studies specifically emphasized the requirement of a humoral immune response to the Her2 TAA [27]. An important role of anti-Her2 antibodies has clearly been shown by the therapeutic effect of Trastuzumab in patients with Her2-positive breast cancer

[49]. Considering these reports and our results, it appears most promising to combine different immunotherapy regimens such as the here-presented T cell stimulating replicon approach with the already established therapeutic Her2 antibody Trastuzumab to achieve synergistic effects.

Acknowledgments

We thank Dr. E.M. Jaffee for the NT-2 cell line, Dr. K.L. Rock for the DC2.4 cell line and Dr. N. Tautz for the anti BVDV NS3 antibody. This study was supported by the Martin-Luther-University Halle-Wittenberg and the intramural research program of NIDDK, NIH.

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Fig. 1.

Schematic representation of BVDV replicons and Her2 fragments. (A) Organization of mono- and bi-cistronic BVDV replicon constructs. The monocistronic replicon DI9c, a minimal truncated version of the BVDV genome [14], consists of the 5' - and 3' untranslated regions (UTRs; indicated by lines) and the coding region of the viral proteins N^{pro}, NS3, NS4A, NS4B, NS5A and NS5B (indicated by boxes). Following entry into the cell, the viral RNA first acts as an mRNA. Translation is mediated by an internal ribosomal entry site (IRES) in the 5'UTR and leads to the synthesis of a polyprotein that is processed by viral proteases. The NS proteins and host proteins form the viral replicase which amplifies the viral RNA in the cytoplasm via negative-strand RNA intermediates [50]. In the bi-cistronic replicons (see text), heterologous ORFs encoding either rat Her2 fragments or mouse IL-12 were inserted downstream of the BVDV 5'UTR and expressed as fusion proteins with N^{pro} and a FLAG (flagellin) epitope. Translation of the NS-proteins is mediated by the encephalomyocarditis virus (EMCV) IRES. (B) Domain organization of the rat Her2 fragments. Rat Her2 (*rHer2*) is composed of an extracellular domain (ECD) of approximately 600 amino acids, a transmembrane segment (TM, horizontal lines) and an intracellular domain (ICD) of roughly 500 amino acids. The locations of H2^d-restricted CD8 T cell epitopes are indicated as bold vertical lines [25,51,52].

Fig. 2.

Evaluation of replicon properties. (A) Cell death. The figure shows a representative flow cytometry experiment performed with the *rHer2*-MF replicon. Numbers in the upper two quadrants of each dot plot indicate the percentage of dead cells, numbers in the right quadrants indicate the percentage of BVDV NS3-expressing cells. (B) Protein expression of Repl-*mIL12*. Left panel: western blot analysis performed with an anti-Flag antibody and proteins that derived from cell lysates of mock-transfected (transfected in the absence of exogenous RNA) and Repl-*mIL12*-transfected MDBK cells, respectively. The arrow marks a protein band that has the theoretical molecular weight of *mIL-12* (61 kDa). Right panel: detection of IL-12 in the cell culture supernatant of Repl-*mIL12*-transfected DC2.4 by EIA. Mean values of results from three independent experiments are shown ($\pm$ standard error of the mean). Supernatants from DC2.4 cultures transfected with *rHerMF* did not contain IL-12 (data not shown). (C) Protein expression of Repl-*rHer2*MF and Repl-*rHer2*ECD. The phosphorimager gel image shows [³⁵S]-labeled proteins produced in an in vitro translation-reaction performed with the indicated replicons (12% SDS-PAGE). While the most prominent BVDV proteins NS3 and N^{PRO} were translated from all tested replicon RNAs, specific additional bands indicated the expression of the heterologous proteins by the bi-cistronic Repl-*rHer2*MF and Repl-*rHer2*ECD RNAs. The molecular weights of these additional protein bands corresponded to those of the encoded Her2 protein fragments (indicated) and to precursors consisting of the Her2 protein fragments and N^{PRO} (not indicated), respectively.

Fig. 3.

Vaccination with Her2 replicon-transfected DCs protects mice against tumor challenge. (A) Percentage of tumor-free mice. The percentage of tumor-free mice was determined 21 days after tumor challenge in groups of mice that were vaccinated with DC2.4 transfected with either single replicons (groups ECD, MF, IL-12) or with a combination of Her2-replicons plus Repl-*mIL12* (ECD + IL-12, MF + IL-12) or with mock-transfected DC2.4 (see also text). Number of mice per group: mock: $n = 16$, IL-12: $n = 10$, ECD: $n = 12$, ECD + IL-12: $n = 8$, MF: $n = 8$, MF + IL-12: $n = 14$. Mice vaccinated with the Her2 replicons were significantly protected against tumor growth as indicated by the percentage of tumor-free mice in each group 21 days after tumor challenge. (B) Mean tumor sizes. The average tumor sizes were determined over 21 days after tumor challenge for the different groups of mice (see A and text). Mean values $\pm$ standard error of the mean are shown. Tumors of the following mouse groups were significantly smaller than tumors of the mock-vaccinated control group ($p < 0.05$): day 9, ECD + IL-12, MF + IL-12; day 12, MF, IL-12, ECD + IL-12, MF + IL-12; day 14, MF, ECD + IL-12, MF + IL-12; day 18, ECD, MF, ECD + IL-12, MF + IL-12; day 21: ECD, MF, ECD + IL-12, MF + IL-12. Note that one of 12 mice in the ECD group was excluded from the analysis of the average tumor size because the tumor was flat and thus tumor volume was overestimated by the measurement of only two dimensions. (C) Mean tumor size on day 21 after tumor challenge (data points obtained with the individual mice). Mice vaccinated with Her2 replicons developed significantly smaller tumors compared to mock-vaccinated mice after tumor challenge (ECD: $p = 0.0086$, MF: $p = 0.0127$, ECD + IL-12: $p = 0.011$; MF + IL-12: $p = 0.001$).

Fig. 4.

The vaccination-induced anti-tumor effect is mediated by T cells. All mice were vaccinated with Repl-*r*HerMF-transfected DCs and Repl-*m*IL12-transfected DCs and either left untreated (undepleted) or were injected with CD4- or CD8-depleting antibodies before and during tumor challenge (CD4-depleted or CD8-depleted, respectively; see Section 2). (A) Representative FACS dot plots of splenocytes from undepleted and depleted mice on day 22 post tumor challenge. Splenocytes were pregated for lymphocytes, followed by the exclusion of B cells and dead cells. (B) Mean tumor size ($\pm$ standard error of the mean) of each vaccination group measured over 22 days after tumor challenge. (C) Mean tumor size on day 22 after tumor challenge (data points obtained with the individual mice). All mice in the depletion groups developed tumors whereas four out of six mice remained tumor-free (tumor volume of zero) in the control group. Number of mice per group: $n = 6$.

Fig. 5.

Her2-specific CD8 T cells are induced by cross-priming. (A) Splenocytes from H-2^q+ FVB/N mice that had been vaccinated either with Repl-*r*Her2ECD-transfected H-2^b+ DC2.4 (left panel) or with a combination of Repl-*r*Her2ECD and Repl-*m*IL12-transfected DC2.4 (right panel) were tested on day 21 after tumor challenge in IFN- γ ELISpot assays against H-2^q+ 3T3 cells pulsed with overlapping Her2 peptides (see Section 2). The number of IFN- γ spot forming units (SFU) is indicated. Positive responses, defined as ≥ 100 SFU per 1.25×10^5 CD8 T cells, are indicated in red for each individual mouse. The tumor size of each mouse is shown in the graph on the right of each ELISpot graph. (B) Repl-*r*Her2MF-transfected DC2.4 (left panel) or a combination of Repl-*r*Her2MF plus Repl-*m*IL12-transfected DC2.4 (right panel) were used for vaccination. (C) Mock-transfected DC2.4 (left panel) or Repl-*m*IL12-transfected DC2.4 (right panel) were used for vaccination. (D) Correlation between tumor volume and IFN- γ SFU (mean SFU from 1.25×10^6 and 0.63×10^6 CD8 T cells) of each mouse (filled circles). Data from all vaccination groups were included. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6.

Vaccination does not induce Her2-specific antibodies. (A) Detection of Her2-specific antibodies. Flow cytometry histograms of 3T3 (grey line) and Her2-expressing 3T3/neu cells (black line). The cells were incubated with plasma from mock-vaccinated mice prior to tumor challenge (negative control, left panel) and with plasma from mock-vaccinated mice 21 days after tumor challenge (positive control, right panel), respectively, and stained with a secondary, PE-labeled antibody. (B) Representative histograms obtained with the plasma of vaccinated mice, the vaccination regimen is indicated above each histogram. (C) Relative Her2 antibody concentrations defined by the ratio of the mean fluorescence intensity (MFI) of 3T3/neu vs. 3T3 cells. None of the vaccinated mice displayed a higher relative antibody concentration than the mock control showing that the vaccination did not induce Her2-specific antibodies. The MFI(3T3/neu)/MFI(3T3) ratio for the positive control shown in (A) was 32. Number of mice tested per group: mock: $n = 7$, IL-12: $n = 3$, ECD: $n = 4$, ECD + IL-12: $n = 4$, MF: $n = 4$, MF + IL-12: $n = 3$.