Dendritic Cells Transfected with Her2-Encoding RNA Replicons Induce Cross-Priming and Protect Mice against Tumor Challenge

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1 Summary

Cancer is a leading cause of death worldwide. Despite the improvement of conventional cancer therapies, the mortality rate remains high and current treatment options are often associated with serious side effects. Thus, immunotherapy has been proposed as a novel approach for cancer treatment. Cancer immunotherapy exploits the highly specific and efficient nature of the immune system to eradicate or prevent tumors. The redirection of the immune system to act against tumor associated antigens can be achieved by loading dendritic cells (DCs) with a tumor antigen. DCs are professional antigen presenting cells that efficiently activate T cells. However, current methods of loading DCs with tumor antigens, ranging from transduction with viral vectors to transfection with peptides, proteins or nucleic acids, are either not safe for application in humans or fail to elicit a satisfying anti-tumor effect.

In this study, transfection of DCs with cytopathic RNA replicons was established as an alternative procedure to express tumor antigens in DCs. To this end, recombinant RNA replicons of bovine viral diarrhea virus (BVDV) were applied. BVDV replicons encoding foreign antigens replicate at high levels in the transfected cells and thus enable effective production of the antigen. Moreover, RNA replication induces DC apoptosis, a process that was previously shown to promote cross-priming (Racanelli et al., 2004). Cross-priming defines the transfer of cell-associated antigens from apoptotic, replicon-containing cells, to antigen presenting cells, which than prime CD8+ T cells.

Using the breast cancer antigen Her2 as a model tumor associated antigen, two replicons were constructed that encode either the extracellular domain (ECD) or the middle fragment (MF) of Her2. In addition, a replicon was generated that expresses the mouse interleukin 12 (mIL-12) gene. Murine DC2.4 dendritic cells (haplotype H-2^b) were transfected with individual Her2 replicons or with combinations of Her2 and mIL-12 replicons respectively, and FVB/N mice (haplotype H-2^q) were vaccinated twice with these cells. To test for a preventive anti-tumor effect, the mice were subsequently challenged with Her2-expressing breast cancer cells, and tumor growth was measured thrice weekly for three weeks. Vaccination with replicon-loaded DCs resulted in significantly smaller tumors compared to the mock-vaccinated control group. Furthermore, up to 75% of the vaccinated mice remained tumor-free, whereas all mock-vaccinated mice developed tumors. However, therapeutic vaccination, i.e. injecting replicon-transfected DCs after tumors had already established had no effect.

In depth analysis of the immune response that protected mice against tumor growth after preventive vaccination revealed that the anti-tumor effect correlated with a T cell response that was induced by cross-priming. That is, depletion of CD4+ or CD8+ cells prior to tumor challenge abrogated the anti-tumor effect. Moreover, analysis of the CD8+ T cell response demonstrated that T cells were induced by cross-priming,

because the lymphocytes produced IFN-γ in response to peptide-loaded cells from the FVB/N mouse (H-2^q) background. Notably, antibody responses against Her2 were not detected in the vaccinated mice suggesting that the *in vivo* effect of the vaccination was solely dependent on T cells.

In conclusion, this study demonstrates that cytopathic RNA replicons expressing a TAA can be used as a tool to mediate a preventive anti-tumor effect via the induction of T cell cross-priming. These results emphasize the potential of cross-priming as a vaccine strategy. Lastly, this approach may be applicable as a safe and efficient means to load DCs with a tumor antigen in cancer immunotherapy.

2 Introduction

2.1 Cancer and cancer treatment

Cancer is one of the major causes of death worldwide and about one in three people in developed countries will be diagnosed with the disease during their lifetime (Rheingold et al., 2003). In 2007, approximately 7.6 million people died of cancer worldwide (American Cancer Society).

The transformation of cells, i.e. the process of acquiring malignant properties, resulting in uncontrolled proliferation, the ability to invade neighboring tissues and, eventually, other parts of the body (metastasis), defines the disease cancer. Any type of tissue can be affected and it occurs in patients of any age, although predominantly in individuals over 65 years (Watson et al., 2006).

Mutations in two groups of genes mediate the transformation of cells, namely gain of function mutations in proto-oncogenes and loss of function mutations in tumor-suppressor genes. Proto-oncogenes encode for proteins that are involved in cell growth and differentiation, whereas tumor-suppressor genes protect cells from malignant transformation or induce apoptosis of damaged cells. Multiple factors can induce those mutations, including genetic predisposition, environmental factors or viral infections (Frei et al., 2003). Subsequently, the accumulation of these mutations induces the changes that progressively transform normal cells into malignancies. Hanahan and Weinberg (2000) described seven steps that are involved in this transformation: (i) Self-sufficiency in growth signals, (ii) insensitivity to anti-growth signals, (iii) tissue invasion, (iv) metastasis, (v) limitless replicative potential (immortality), (vi) sustained angiogenesis and (vii) the evasion of apoptosis.

In order to treat cancer, every malignant cell must be eradicated, because only a few surviving cells can initiate the formation of a new tumor (Marlo et al., 2006). To date, the predominant treatment modalities are surgery, radiotherapy and chemotherapy. Surgery aims at the complete excision of malignant tissue or the entire organ and is mainly used for the treatment of solid tumors. However, this method is ineffective if the tumor cells have already spread to other tissues. Radiotherapy uses ionizing radiation to destroy tumor cells. Despite the fact that healthy cells are supposed to recover from radiation exposure, damage to non-malignant cells cannot be avoided. Most side effects occur locally at the site of radiation; however, chronic toxicity can result from high dose radiotherapy. Chemotherapy is the administration of cytotoxic drugs that target rapidly dividing cells. The therapy is not specific to cancer cells because some healthy tissue cells, such as bone marrow cells, also have a high proliferation rate. Chemotherapy often causes significant toxicities because the drug must be administered at high doses. Consequently, the treatment can have severe side effects

like cardiotoxicity, sterility and immunosuppression. Yet, chemotherapy is the treatment of choice in about 60–70% of all patients (Watson et al., 2006).
In summary, conventional cancer therapy is associated with considerable side effects. Furthermore, cancer lethality has only slightly decreased within the last 50 years. For instance in the United States the rate dropped by only 5% between the years 1955 and 2005 (numbers adjusted for size and age of the population, National Center for Health Statistics). Thus, developing or improving new therapies for cancer treatment remains an important research topic.

2.2 Cancer Immunotherapy

Cancer immunotherapy aims at exploiting the immune system for eradicating tumor cells or preventing tumor formation (Baxevanis et al, 2009; Lollini et al., 2006). The main advantage of immunotherapy compared to conventional cancer treatments is the high specificity of the immune system (see 2.2.1). In theory, neoplastic cells could be selectively eliminated without impacting healthy tissue. Furthermore, it might be possible to eradicate malignancies down to the last cell, which would prevent newly arising tumors or metastases. Conversely, another possible application of cancer immunotherapy is the prevention of the disease similar to vaccinations against pathogens.
Similar to vaccinations against viruses and bacteria, cancer immunotherapy can be either active or passive. Active vaccination stimulates the immune system against specific antigens whereas passive vaccination administers antigen-specific effector cells or molecules such as antibodies. Some of the therapies applying antibodies specifically directed against tumor antigens have been approved by the U.S. Food and Drug Administration (Samaranayake et al., 2009), e.g. the monoclonal IgG1 humanized antibody directed against the epidermal growth factor receptor 2 protein (Her2), Trastuzumab, to treat breast cancer (Robertson, 1998).
In contrast to the transient effects of passive vaccination, active vaccination is capable of inducing immunologic memory against tumor antigens. Moreover, active vaccination takes advantage of several effector mechanisms characterizing the immune system (see 2.2.2). Multiple clinical trials are ongoing to test different types of vaccines, such as the administration of DNA or RNA, recombinant viruses encoding tumor antigens, genetically engineered tumor cells or dendritic cells loaded with tumor antigen epitopes (see 2.2.3) (ClinicalTrials.gov). To date, the only vaccinations that might be described as active cancer immunotherapy are those directed against oncogenic viruses, namely human papilloma virus or hepatitis B virus (Jones et al., 2007; Chang et al., 2009).
In the following sections, the requirements for generating an effective immune response against cancer antigens will be discussed in detail.

2.2.1 Immune surveillance

The immune system protects a multicellular organism against pathogens or pathogenic substances. The first response against invading pathogens is mediated by the innate immune system, which is followed by the action of the adaptive immune system. The innate immune system reacts rapidly to infections and provides a first barrier against pathogens. Further, the innate immune response activates the adaptive immune system. The major characteristics of the adaptive immune system are specificity and memory. Both are achieved by a complex interplay of multiple cell types and their products executing specialized functions (Abbas et al., 2007).

Professional antigen presenting cells (APCs) scan the body and take up antigens, which are then processed intracellulary. The processed antigens are subsequently presented on the cell surface bound to major histocompatibility complexes (MHC). As a consequence of antigen uptake, APCs migrate to specialized immune compartments, the secondary lymphoid organs (lymph nodes and spleen), where they present the antigens to T cells (Figure 2.1).

T lymphocytes are defined by the expression of the T cell receptor (TCR) that can recognize specific peptide-MHC-complexes. The repertoire of TCRs is highly diverse due to gene rearrangements and it is estimated that as many as 10^7 different TCR specificities exist in each individual. When the T cell recognizes a peptide-MHC-complex on APCs, it becomes activated and starts proliferating (“clonal expansion”). The effector function depends on the T cell type: cytotoxic T lymphocytes (CTLs, CD8+ T cells), which are defined by the expression of the CD8 co-receptor, induce apoptosis of cells presenting antigen peptides on MHC I molecules; in contrast, T helper cells (T_H, CD4+ T cells) that are characterized by CD4 expression, provide help to other immune cells by delivering activating or co-stimulating signals. For instance, CD4+ T cells support the development of memory CD8+ T cells. T_H cells recognize antigen peptides bound to MHC II molecules (Abbas et al., 2007).

Each cell, regardless of type and tissue, presents its antigen content in the form of peptides bound to MHC class I molecules on the cell surface, where they can be recognized by CD8+ T cells. Several mechanisms exist to prevent an immune reaction against self-antigens (auto-immune reactions) to avoid the destruction of healthy, uninfected cells.

Cancer cells differentiate from healthy cells and therefore express large portions of the normal set of antigens. Nevertheless, neoplastic cells may differ in their antigen expression pattern as a consequence of the transformation to uncontrolled cell growth and the acquirement of the ability to invade other tissues. Thus, cancer cells are subject to recognition by the immune system, a concept described as immune surveillance (Burnet, 1970).

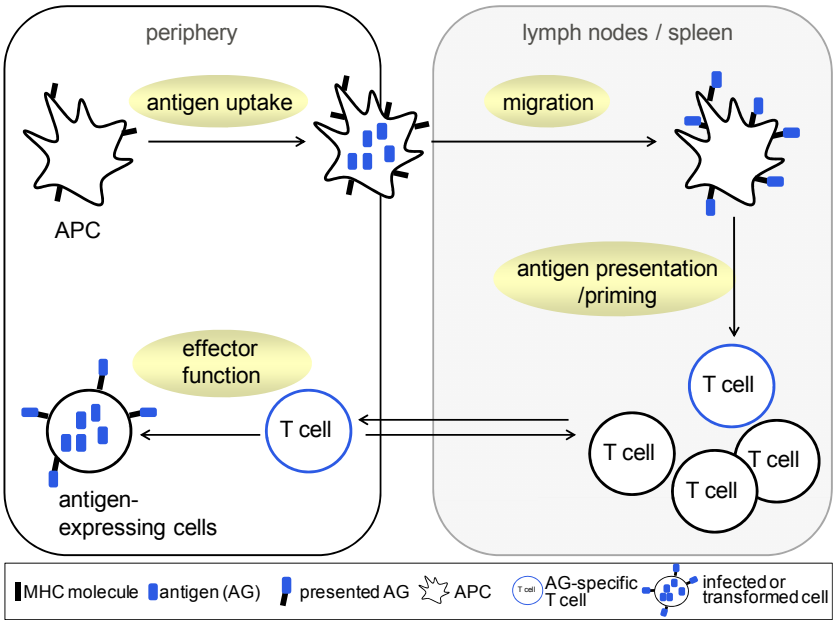

Figure 2.1: Role of antigen presenting cells (APCs) in the activation of the adaptive immune response

As a consequence of antigen uptake in peripheral tissues (white area), APCs migrate to the secondary lymphoid organs, the lymph nodes and spleen (grey area), and present antigen peptides on MHC molecules. In this specialized lymphoid tissue, APCs come in contact with T cells. Antigen-specific T cells are activated by the APCs and consequently migrate to the peripheral tissue to mediate an effector function upon encounter of antigen-expressing cells.

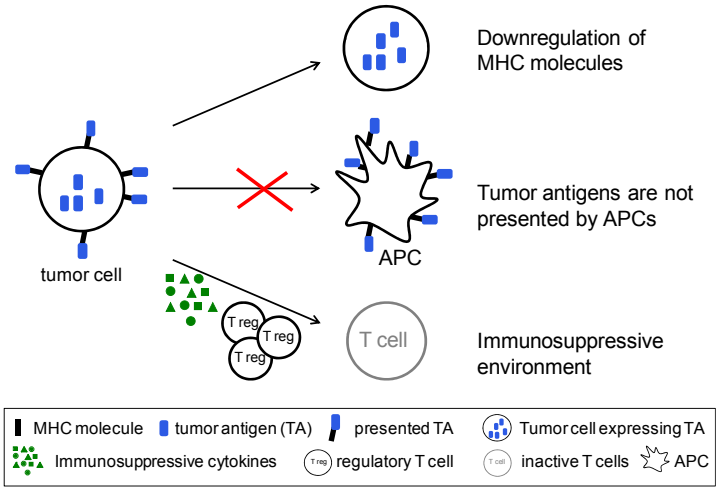

Figure 2.2: Immune evasion mechanisms of tumor cells

Some tumors downregulate the expression of MHC I molecules to prevent recognition by CD8+ T cells. Another observed mechanism to escape immune surveillance is the unavailability of tumor antigens for APCs, thus inhibiting T cell priming. Tumors can secrete immunosuppressive cytokines or favor the activation of regulatory T cells (T reg), while both mechanisms dampen the effector functions that would lead to the eradication of transformed cells.

However, the high incidence of cancer implies that the immune surveillance system is not foolproof. The reason for this situation is that the development of tumor cells underlies a permanent selection process one of the determinants of which is low immunogenicity to evade the immune system (Shankaran et al., 2001). This is achieved, for instance, by down-regulation of MHC molecules on the cell surface (Garrido et al., 1993), the lack of antigen availability for APCs or by a specifically created immunosuppressive environment which renders the immune system tolerant against the tumor antigens (reviewed by Khong & Restifo, 2002)(Figure 2.2).

2.2.2 Effective immune response against cancer cells

Successful immunotherapy breaks the tolerance against tumor antigens. Along this line, several components of the immune system can be exploited for cancer immunotherapy. Predominantly, vaccination strategies aim at inducing an adaptive immune response against neoplastic cells (Boon et al., 2006). Specific targeting of tumor cells is crucial for cancer immunotherapy to avoid killing of healthy cells. Therefore, immune responses are induced against so-called tumor-associated antigens (TAA). TAAs are proteins that are mainly expressed by tumor cells and are present on only a few normal cells or expressed at low levels. Additionally, TAAs play a role in tumorigenesis.

CD4+ T cells were described to play an important role in the response against tumors (reviewed by Gerloni & Zanetti, 2005). T_H cells have various effector functions that can directly or indirectly execute an anti-tumor function. In particular, T_H cells provide an immunostimulatory cytokine milieu that attracts cells of the innate immune system (Tsung et al., 2002), activate APCs (Fruh & Yang, 1999) and provide help for CD8+ T cells by mediating their activation and persistence (Giuntoli et al., 2002). Moreover, type 1 T_H (T_H1) cells are required for the development of memory T cells. T_H1 cells produce cytokines such as interferon γ (IFN-γ) and activate macrophages, which, in turn, can non-specifically kill tumor cells (Abbas et al., 2007). Additionally, IFN-γ exhibits direct anti-tumor effects such as enhancing antigen processing and presentation and inhibiting tumor growth mediated by its anti-angiogenic effect (Qin & Blankenstein, 2000)(Figure 2.3).

CD4+ T cells of the T_H type 2 (T_H2) activate B cells to produce antibodies against the targeted antigen. The role of antibodies in cancer is diverse; on the one hand, an increasing antibody titer in cancer patients is associated with a poor prognosis (Houbiers et al., 1995), but on the other hand the passive administration of antibodies has been shown to have anti-tumor activity (Riethmuller et al., 1999). Antibodies can mediate the killing of target cells by different mechanisms, such as antibody-dependent cellular cytotoxicity or the activation of the complement system. In addition to the activation of immune mechanisms, antibodies can have a direct effect on tumor cells if the targeted antigen is required for tumorigenicity (Figure 2.3).

T_H17 cells have been recently described as a third lineage of CD4+ T cells (Harrington et al., 2005) that are involved in the host defense against pathogens as well as in autoimmune diseases (reviewed by Korn et al., 2009). First studies indicate an involvement of T_H17 cells in the response against malignant cells (Muranski et al., 2008; Derhovanessian et al., 2009), however, so far, the role of T_H17 cells in the anti-tumor response is poorly understood (Muranski & Restifo, 2009).

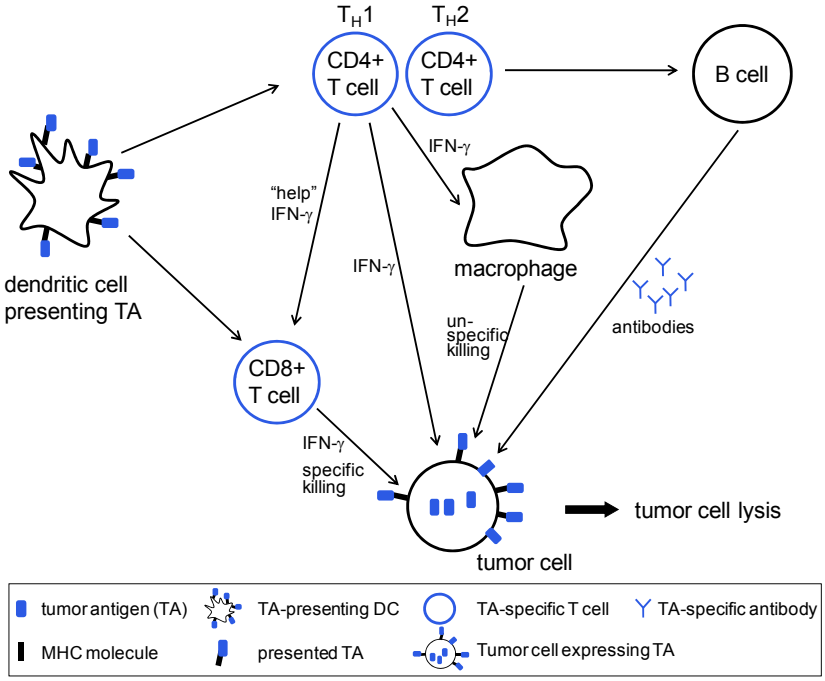

Figure 2.3: Anti-tumor effector mechanisms of cells of the adaptive immune system

APCs present tumor antigens and activate CD8+ and CD4+ T cells. Activated CD8+ T cells secrete IFN-γ and directly kill cells expressing the antigen. CD4+ T cells of the T_H1 type provide help for CD8+ T cells and produce IFN-γ that, in turn, activates macrophages to unspecifically kill tumor cells. CD4+ T cells of the T_H2 type activate B cells to produce tumor antigen-specific antibodies that mediate killing of the target cells by antibody-dependent cellular cytotoxicity or the complement system.

Undoubtedly, CD8+ T cells are important effector cells for a successful anti-tumor response (Figure 2.3). Thus, tumor infiltrating CD8+ T cells have been associated with a better prognosis (Clemente, et al., 1996), and adoptive transfer of CD8+ T cells is highly effective against solid tumors, e.g. in melanoma patients (Rosenberg & Dudley, 2009). CD8+ T lymphocytes recognize antigens independently of their location in the cell, because they bind to peptides presented on MHC I molecules on the cell surface. Upon recognition of a target cell, CD8+ T cells kill the cell by inducing apoptosis via the secretion of perforin and granzymes or expression of the Fas ligand (Abbas et al., 2007). Furthermore, CD8+ T cells also produce IFN-γ, which renders tumor cells more susceptible to killing by CD8+ T cells as a consequence of increased antigen presenta-

tion. Because of the direct killing ability and the intracellular nature of most tumor antigens, CD8+ T cells are a crucial part of the immune response induced by cancer immunotherapy.

2.2.3 Dendritic cells and their use for immunotherapy

Dendritic cells (DC) are professional APCs. They take up and process antigens from peripheral tissues for the purpose of presentation on MHC class I and II (Abbas et al., 2007). After acquiring the antigens, DCs mature and migrate to the draining lymph nodes where they activate CD4+ and CD8+ T cells. The mature DC phenotype is characterized by high expression of MHC and co-stimulatory molecules. DCs are highly efficient in priming an immune response against viruses, microorganisms and tumors. Because of their specialized role in antigen presentation and T cell activation, DCs are ideal for the use in immunotherapy. Hsu (1996) published the first clinical trial using autologous peptide-pulsed DCs for the vaccination of patients with B-cell lymphoma and showed an induction of the cellular immune response as well as a partial clinical response. Since then, multiple trials have been pursued and the underlying procedure has been shown to be safe, well-tolerated in humans and to induce immune responses in patients (Taken et al., 2007). In the last decade, the knowledge in the field has significantly increased, in particular concerning the preparation of autologous DCs and the ideal maturation status of these cells for immunotherapy. Remarkably though, only a few complete responses and only some partial responses were reported in clinical studies (Lollini et al., 2006, Engell-Noerregaard et al., 2009).
The effectiveness of DC immunotherapies is mainly determined by the elicited immune response. In order to induce a strong immune response, (i) high antigen concentrations are needed and (ii) activation of DCs must occur to induce an immune state against the tumor rather than tolerance (Schuler et al., 2003; Melief, 2008). Both factors are predominantly determined by the antigen loading method (Gilboa, 2007). Along this line, the choice of the DC loading method is of major importance for the potency of the vaccination, as it determines the efficiency of antigen presentation on both MHC I and MHC II molecules and ultimately the degree of stimulation of the immune system. Various methods of loading DCs with tumor antigens have been studied, including (i) transfection with the protein-antigen or antigen-derived peptides, (ii) transfection or transduction of DCs with antigen-encoding DNA or RNA or (iii) loading DCs with tumor lysate or whole tumor cells (reviewed by Tuyaerts et al., 2007; Osada et al., 2006).

2.3 Cytopathic bi-cistronic BVDV replicon

A study by Racanelli et al. (2004) demonstrated that cytopathic so-called “bi-cistronic BVDV replicons” can be used to load DCs and that vaccination with such modified DCs can be applied to induce a strong immune response against the hepatitis C virus (HCV) antigen NS3 (see 2.3.1). Cytopathic bi-cistronic replicons are single stranded RNA molecules that consist of two open reading frames (ORF). The viral RNAs replicate autonomously and mediate apoptosis in transfected cells (Figure 2-5).

The sequence of the replicon RNA is derived from the genome of the Bovine Viral Diarrhea Virus (BVDV). BVDV is a pathogen that may cause mucosal disease in cattle and that belongs to the genus *Pestivirus* in the family *Flaviviridae*. BVDV is a positive-strand RNA virus, i.e. the viral genome operates directly as a messenger RNA (mRNA) following entry into the host cell. The BVDV genome encodes one ORF that is flanked by un-translated regions (UTRs) at the 5’ and 3’-ends (Figure 2.4) (reviewed by Lindenbach et al., 2007). An internal ribosome entry site (IRES) mediates translation initiation resulting in a polyprotein that is cleaved by viral and host proteases (Poole et al., 1995). Non-structural (NS) proteins of BVDV (NS2/3, NS4A, NS4B, NS5A, NS5B) together with host factors form a replication complex in which NS5B exhibits RNA-dependent RNA polymerase activity. As with other positive-strand RNA viruses, the genome is replicated via negative-strand RNA intermediates (Zhong et al., 1998).

It was shown that subgenomic viral RNAs that encode solely the 5’ and 3’ UTR, the autoprotease N^{PRO} and the BVDV NS proteins NS3, NS4A, NS4B, NS5A and NS5B contain all RNA elements and viral protein coding regions that suffice to catalyze viral RNA replication in the cytoplasm of transfected host cells (Behrens et al., 1998). The autonomously replicating RNA is consequently referred to as “BVDV replicon”. Tautz et al. (1999) subsequently developed a bi-cistronic construct of the replicon by inserting an additional IRES element of encephalomyocarditis virus (EMCV) upstream of the NS-coding regions and an additional heterologous ORF downstream of the BVDV IRES. Thus, while translation of the heterologous gene is mediated by the BVDV IRES, expression of the viral replicase proteins NS3-NS5B is maintained by the EMCV IRES (Figure 2.4).

There exist two so-called “biotypes” of BVDV, namely a cytopathogenic and a non-cytopathogenic form (McClurkin et al., 1985). The difference between the two forms concerns the activity of an autoprotease that mediates the proteolytic cleavage between NS2 and NS3. Differences in this autoprotease activity and, consequently, differences in the level of intracellular NS3 are known to originate from insertions of viral or foreign RNA sequences during BVDV replication (Meyers et al., 1991). For example, an insertion of 27 nucleotides of unknown origin into the NS2 coding region was shown to generate a polyprotein that showed a significantly higher level of the NS2-3 autoprotease (Tautz et al., 1994; Lackner et al., 2007). Increased levels of NS3

correlate with a cytopathogenic phenotype; specifically, it was shown that NS3 may increase the activity of caspase 9 and caspase 3 leading to the induction of apoptosis (Gamlen et al., 2009). The induction of apoptosis is described as the cytopathic effect. Cytopathic replicons express high levels of NS3, simply because they express solely NS3, and when transfected into human, murine or bovine cells in tissue culture cause a moderate cytopathic effect, i.e. they cause cell death within 24 to 48 h post transfection (Figure 2-5).

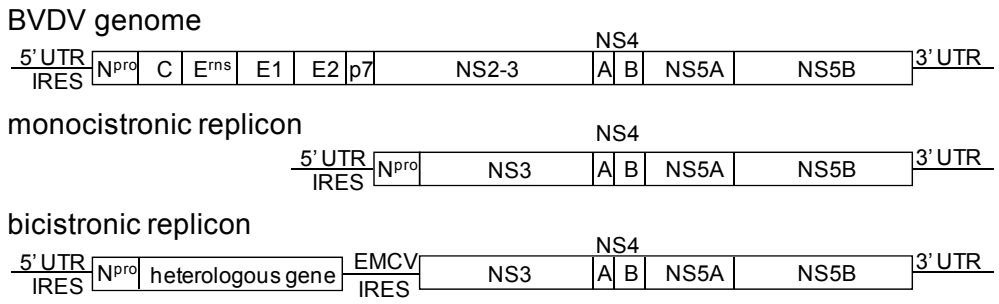

Figure 2.4: Organization of the BVDV genome, the mono-cistronic and bi-cistronic replicon

The figure was adapted from Behrens et al. (1998) and Tautz et al. (1999). BVDV: bovine viral diarrhea virus; UTR: untranslated region; IRES: internal ribosome entry site; N^{PRO}: autoprotease; C, E^{rns}, E1, E2: structural proteins of BVDV; p7: protein encoded by BVDV; EMCV: encephalomyocarditis virus; NS: non-structural proteins of BVDV

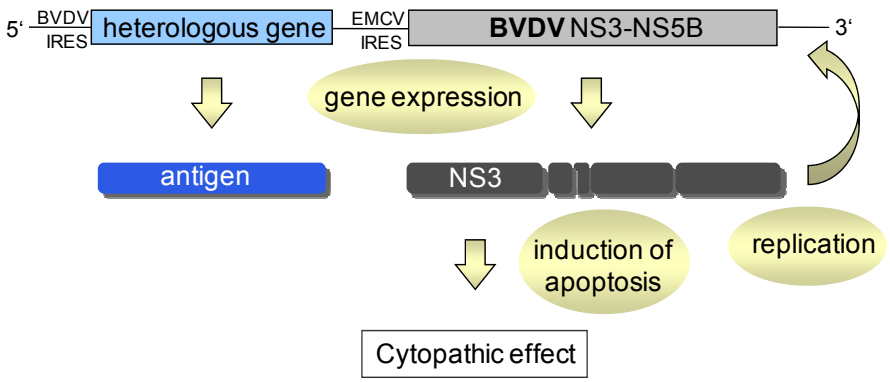

Figure 2.5: Activities of bi-cistronic BVDV RNA replicons in transfected cells

The NS3-NS5B coding regions of the replicon are expressed in the cell providing the components of the replication complex. Consequently, RNA replication mediates (i) high expression levels of the encoded heterologous gene and (ii) production of NS3, in turn, correlates with the induction of apoptosis of the transfected cell. In addition, expression of a heterologous gene yields high amounts of a foreign protein that may serve as an antigen.

2.3.1 Vaccination with DCs transfected with cytopathic bi-cistronic BVDV replicons in an infection model

Racanelli et al. (2004) exploited the intrinsic properties of the cytopathic bi-cistronic BVDV replicons, such as (i) high expression of the heterologous antigen due to amplification of the translation template (replication) and (ii) induction of apoptosis in the transfected cells, to induce an immune response against a virus antigen in an infection model.

The authors constructed a bi-cistronic replicon encoding the HCV NS3 protein and transfected the RNA into the DC line DC2.4. The replicon mediated a high expression level of the HCV NS3 protein and induced apoptosis within 24 h to 48 h following transfection (Figure 2-5). The vaccination of mice induced a strong HCV NS3-specific immune response composed of CD8+ and CD4+ T cells. Of note, the CD8+ T cells were primed by direct and cross-priming (see 2.3.2 Cross-priming) of the HCV antigen. Moreover, the vaccination protected mice against a challenge with an HCV NS3-expressing vaccinia virus.

By comparing a cytopathic and non-cytopathic form of the replicon, Racanelli et al. showed that the cytopathic effect of the replicon is crucial to induce a strong CD8+ T cell response since only the vaccination with the cytopathic replicons protected mice against challenge with an HCV-NS3-expressing vaccinia virus. The reduced immunostimulatory effect of the non-cytopathic replicon was in part rescued by co-transfection of the DCs with a cytopathic, antigen-unrelated replicon. The authors further demonstrated that the cytopathic effect of the replicon promotes cross-priming. Specifically, they showed that fragments of the apoptotic DCs, that were transfected with the cytopathic replicons, were transferred to host APCs in the lymph nodes. Taken together, the cytopathic effect of the replicons induces apoptosis in transfected cells resulting in the uptake of these cells by host APCs, targeting the antigen in the cross-presentation pathway and thereby enhancing the immune response against the antigen (Figure 2.6).

Whereas the study of Racanelli et al. demonstrated that vaccination with replicon-transfected DCs induced a T cell response against the vaccine antigen, the prophylactic and therapeutic *in vivo* effect of the vaccination method could not be adequately addressed because of the lack of a mouse model of HCV infection.

2.3.2 Cross-priming

As outlined earlier, CD8+ T cells recognize antigen peptides bound to MHC I and thereby recognize infected or transformed cells. To execute their effector functions, CD8+ T cells require prior activation by APCs that present the antigen on MHC I molecules. According to the classical dogma of antigen-presentation, intracellular antigens are presented on MHC I and antigens acquired from the extracellular space on MHC II molecules. However, a pathway of presenting exogenous antigens on MHC I mole-

cules must exist to induce CD8+ T cell responses against tumor cells and viruses that do not infect APCs. Bevan (1976) first proposed the mechanism of cross-priming that describes the activation of CD8+ T cells specific for exogenous antigens. Indeed, cross-priming was shown to be involved in the induction of an effective anti-viral (Sigal et al., 1999; Jirmo et al., 2009; Hildner et al., 2008) as well as an anti-tumor response (Nowak et al., 2003).

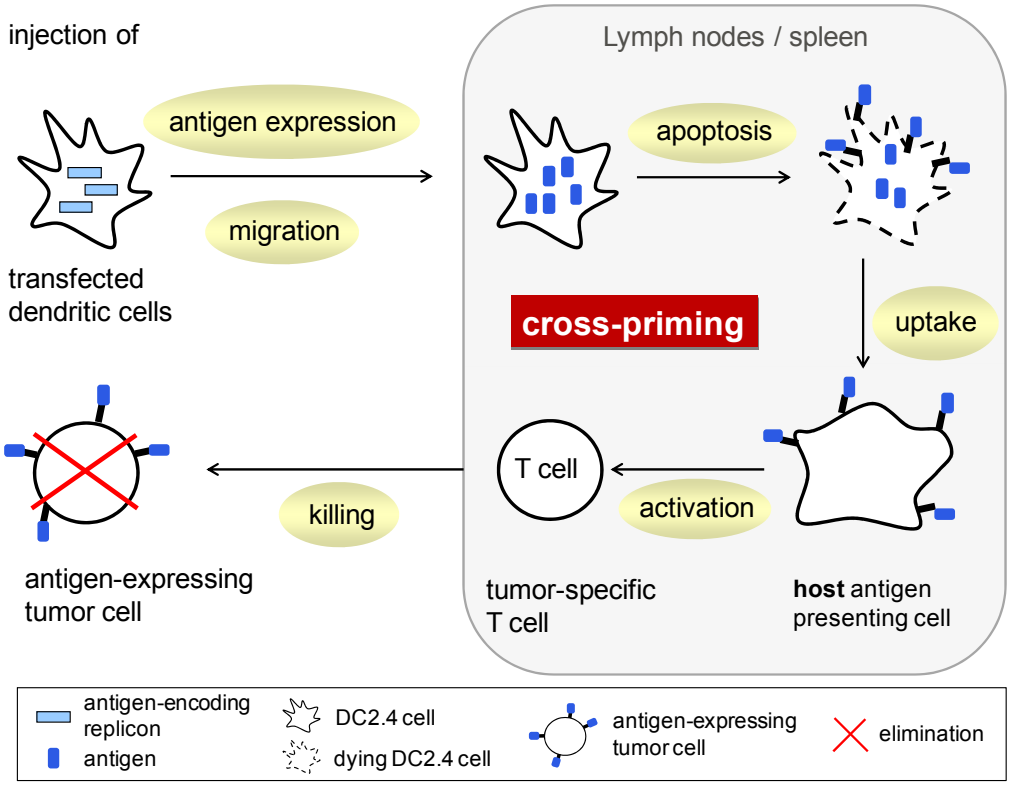

Figure 2.6: Scheme of vaccination with replicon-transfected DCs and cross-priming

Replicon-transfected DCs injected into mice migrate to the secondary lymphoid organs (lymph nodes and spleen) while replication of the RNA mediates expression of high amounts of the encoded antigen. Transfected cells die due to the cytopathic effect of the replicon. This leads to antigen uptake by host APCs, which activate T cells by cross-priming to kill antigen-expressing cells.

Three models evolved that explain the loading of MHC I molecules with exogenous antigens. In the canonical model, the antigen is taken up in phagosomes or endosomes from which it escapes and enters the regular MHC I loading pathway in the endoplasmatic reticlulum (Kurts et al., 1996). Another model describes special vesicles that are formed by the fusion of phagosomes and the endoplasmatic reticlulum that contain MHC I molecules and proteins necessary for antigen presentation (Guermonprez et al., 2003). In a third model, antigens are taken up into stable early endosomes and loaded on MHC I via a mechanism that is independent of the machinery used for presentation of intracellular proteins (Burgdorf et al., 2008).

The predominant cells responsible for cross-priming are CD8α+ DCs (den Haan et al., 2000; Hildner et al., 2008). These cells are present in high numbers in the T cell rich regions of the secondary lymphoid organs, but several other cell types like macrophages (Kovacsovics-Bankowski et al., 1993) and plasmacytoid DCs (Hoeffel et al., 2007) are capable of cross-priming, too.
The source of antigens for cross-presentation may be soluble proteins (Pooley et al., 2001), immune complexes (den Haan & Bevan, 2002), fragments of live cells obtained by cell nibbling (Harshyne et al., 2001), peptides bound to heat shock proteins (Basta et al., 2005) and, in particular, phagocytosed apoptotic cells (Albert et al., 1998, Iyoda et al., 2002). Rawson et al. (2007) showed that proteins cleaved by caspases are direct substrates for the cross-presentation pathway.
Cross-presentation of an antigen leads to priming of CD8+ T cells (cross-priming) or, alternatively, induces tolerance against the antigen (cross-tolerance) (Kurts et al., 1997). Tolerance is likely to be the default setting to maintain self-tolerance, whereas an effector response requires stimulatory signals like Toll-like receptor ligands (Schulz et al., 2005), T cell help (Machy et al., 2002) or type I interferon (Le Bon et al., 2003).
Importantly, cross-priming plays a role in the induction of immune responses via vaccination and it was suggested, that cross-priming might be more important than the direct priming of T cells in this context (Corr et al., 1999; Cho et al., 2001).

2.3.3 The tumor associated antigen Her2

Tumors and viruses are known to evade immune surveillance (Pawlotsky, 2004), in particular the recognition by CD8+ T cells. Considering the promising results achieved by the vaccination against HCV with replicon-transfected DCs (Racanelli et al., 2004), we wanted to evaluate this procedure as immunotherapy against cancer. To specifically target tumor cells, we chose to transfect DCs with replicons encoding a TAA.
A well-studied TAA is Her2 (other synonyms: ErbB2, neu). It is a 185 kDa glycosylated transmembrane protein of the Epidermal Growth Factor Receptor (EGFR) family (Schechter et al., 1984). All members of this family are composed of an extracellular domain consisting of four sub-domains of about 600 residues, a transmembrane segment and an intracellular domain of roughly 500 residues harboring a tyrosine kinase domain (Bagossi et al., 2005) (Figure 4.2). Proteins of the EGFR family dimerize upon ligand binding, which leads to transphosphorylation and subsequent downstream signaling (Schlessinger, 2000).
Her2 plays a role in the development and maturation of the mammary gland and is required for normal cell growth (Schroeder & Lee, 1998). However, over-expression of Her2 induces mammary cancer in transgenic mouse models and is found in 20–25% of primary breast tumors, gastric carcinomas, salivary gland tumors as well as in patients with ovarian cancer (Muller et al., 1988; Owens et al., 2004; Vermeij et al., 2008; Cornolti et al., 2007). The amplification of Her2 is associated with a poor prog-

nosis (Sjögren et al., 1998). Over-expression of the antigen is mainly caused by gene amplification and leads to constitutively active receptor dimers that cause ligand-independent downstream signaling (Liu et al., 1992). Specifically, signaling through the phosphoinositide-3 kinase pathway and the mitogen-activated protein kinase pathway is increased, which promotes cell proliferation, cell-survival, angiogenesis and invasion (Neve et al., 2002; Zhou & Hung, 2003; Westermarck & Kahari 1999).
The current therapy approach to antagonize Her2-associated malignancies is based on the administration of a humanized Her2 antibody, Trastuzumab, in combination with chemotherapy. Trastuzumab is shown to improve disease-free survival in early breast cancer by 52% and the overall survival-rate of patients by 33% (Hudis, 2007).

2.3.4 Interleukin 12

Adjuvants are required to induce a significant immune response and to achieve an immune state rather than tolerance against an antigen delivered by a vaccine (Janeway, 1989; Sasaki & Okuda, 2008; Kyburz et al. 1993). A variety of substances fits this definition, including inorganic and organic substances, microbial components, and, in a broader sense, molecules involved in immunological signaling like co-stimulatory molecules or cytokines.
In order to enhance the immune response mediated by the vaccination with DCs transfected with replicons expressing the tumor antigen, we wanted to test the co-administration of replicons expressing the immuno-stimulatory cytokine interleukin 12 (IL-12) as an adjuvant.
IL-12 plays a key role in the activation of CD8+ T cells and natural killer cells, as well as in the polarization to a T_H1 response (Curtsinger et al, 1999, Kobayashi et al., 1989, Hsieh et al., 1993). These effector functions are either directly mediated by IL-12, or indirectly by the induction of IFN-γ (Manetti et al., 1994).
The above-mentioned qualities make IL-12 well suitable for an application as adjuvant for tumor vaccination strategies (Schmidt and Mescher, 1999). Several preclinical and clinical studies have been performed using IL-12 in combination with peptide or DNA vaccines to immunize against tumors resulting in an increase of the T cell response and promising results regarding the disease outcome (Elzaouk et al., 2006; Hamid et al., 2007; Lee et al., 1999).

2.4 Objective

The objective of this study was to apply DCs transfected with cytopathic replicons for the vaccination against tumor cells. To evaluate the *in vivo* effect of the vaccination with replicon-transfected DCs, vaccination was tested in the Her2 mouse model. Using the murine H-2^b DC line DC2.4 and H-2^q mice, we further evaluated the efficiency of an immune response that was exclusively primed by cross-presentation of the vaccine antigen.

Specifically, the aims were:

- To construct and characterize replicons encoding epitope-rich fragments of the tumor antigen Her2 and murine IL-12.
- To test the application of DCs transfected with replicons for delivering immunostimulatory molecules together with a vaccine antigen.
- To determine the *in vivo* effect of cross-priming induced by vaccination with allogeneic, replicon-transfected DCs by
  - analyzing tumor growth in vaccinated mice (prophylactic vaccine).
  - analyzing tumor development of established tumors after vaccination (therapeutic vaccine).
- To determine the mechanism mediating the anti-tumor effect of the vaccination with replicon-transfected DCs by analyzing
  - the CD8+ T cell response.
  - the CD4+ T cell response.
  - the antibody response.

3 Materials and Methods

3.1 Materials

Table 3.1: Chemicals

CHEMICAL	VENDOR
3-(N-morpholino)propanesulfonic acid (MOPS)	Merck, Darmstadt, Germany
Acrylamide solution; Rotiphorese Gel 30 (acrylamide 38%, bisacrylamide)	Pierce, Rockford, IL, USA
Adenosine triphosphate (ATP)	Roche, Mannheim, Germany
Agarose	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Ammonium persulfate (APS)	Pierce, Rockford, IL, USA
Ammoniumacetate	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Calcium chloride	Merck, Darmstadt, Germany
Creatine phosphate	Roche, Mannheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Disodium phosphate	Roth GmbH, Karlsruhe, Germany
Dithiothreitol (DTT)	Serva, Heidelberg, Germany
Ethidium bromide	Gibco, Carlsbad, CA, USA
Ethidium monoazide	Invitrogen, Carlsbad, CA, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Formamide	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Merck, Darmstadt, Germany
Guanosine triphosphate (GTP)	Roche, Mannheim, Germany
Humilin R, regular recombinant human insulin	Eli Lilly, Indianapolis, IN, USA
LB medium (Luria-Bertani medium)	Gibco, Carlsbad, CA, USA
Magnesium acetate	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Manganese(II) chloride	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Methotrexate	Sigma-Aldrich, St. Louis, MO, USA
N-octylglycopyranoside	EMD chemicals, Darmstadt, Germany
Paraformaldehyde	Sigma-Aldrich, St. Louis, MO, USA

CHEMICAL	VENDOR
Phenol/chloroform/isoamyl alcohol	Roth GmbH, Karlsruhe, Germany
Potassium acetate	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Potassium dihydrogen phosphate	Roth GmbH, Karlsruhe, Germany
Promix 35Met/35Cys	GE Healthcare, Munich, Germany
Rubidium chloride	Roth GmbH, Karlsruhe, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium azide	Sigma-Aldrich, St. Louis, MO, USA
Sodium chloride	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Sodium dodecyl sufate (SDS)	Sigma-Aldrich, GmbH, Deisenhofen, Germany
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Pierce, Rockford, IL, USA
Tris[hydroxylmethyl]aminomethane (Tris)	Sigma-Aldrich, GmbH, Deisenhofen, Germany
TritonX-100	Sigma-Aldrich, St. Louis, MO, USA
Tween20	Sigma-Aldrich, St. Louis, MO, USA

Table 3.2: Buffer solutions

BUFFER	COMPOSITION
Cell lysis buffer	50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100
FACS buffer	PBS, 2.5% FCS, 0.02% NaN_3
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.2 or 7.4
RNA gel buffer	20 mM MOPS buffer, pH 7.0; 5 mM sodium acetate; 1 mM EDTA; 250 mM formaldehyde
TAE	40 mM Tris/HCl, pH 8.3; 1 mM EDTA; 40 mM acetate
TBS	50 mM Tris/HCl, pH 7.4, 150 mM NaCl
TBT	50 mM Tris/HCl, pH 7.4, 150 mM NaCl; 0.1% Tween
Transformation buffer I	30 mM Potassium acetate; 100 mM $RbCl_2$; 10 mM $CaCl_2$; 50 mM $MnCl_2$; 15% Glycerol (v/v); pH 5.8
Transformation buffer II	10 mM MOPS; 10 mM $RbCl_2$; 75 mM $CaCl_2$; 15% Glycerol (v/v); pH 6.5
Western blot buffer	25 mM Tris, 200 mM glycine, 10% methanol

Table 3.3: Enzymes and standards

ENZYMES AND STANDARDS	VENDOR/SOURCE
Creatine kinase	Roche, Mannheim, Germany
DNA-ladder (1 kb)	Fermentas, St. Leon-Rot, Germany
DNase I, RNase-free recombinant, 10,000 U	Roche Applied Sciences, Indianapolis, IN, USA
Klenow fragment	Fermentas, St. Leon-Rot, Germany
Protease inhibitor	Roche Applied Sciences, Indianapolis, IN, USA
Protector RNase inhibitor, 40 U/ml	Roche Applied Sciences, Indianapolis, IN, USA
proteinmarker	Fermentas, St. Leon-Rot, Germany
Restriction endonucleases	NewEnglandBiolabs, Fermentas
Ribonucleoside triphosphate set (rNTP mix), 20 µmol	Roche Applied Sciences, Indianapolis, IN, USA
S10 extract from Huh7 cells	(prepared following the protocol of Barton and Flanegan, 1993) Paul Knick, Martin-Luther-University
SP6 RNA-Polymerase, 20 U/µl	Roche Applied Sciences, Indianapolis, IN, USA
*Srf*I, 10 U/ml	Stratagene, La Jolla, CA
Streptavidin	Invitrogen, Carlsbad, CA, USA
T4 DNA Ligase	Fermentas, St. Leon-Rot, Germany
T7 RNA polymerase	Stratagene, La Jolla, CA

Table 3.4: Antibodies

ANTIBODIES	VENDOR
Anti-Flag M2© F3165	Sigma-Aldrich, GmbH, Deisenhofen, Germany
Anti-GAPDH-antibody	Abcam, Cambridge, MA, USA
Anti-Her2 antibody; clone 7.16.4	Calbiochem, San Diego, CA, USA
Anti-mouse-CD4-APC/Cy7; clone L3T4	BD Biosciences, San Jose, CA, USA
Anti-mouse-B220-PE/Cy5; clone RA3-6B2	BD Biosciences, San Jose, CA, USA
Anti-mouse-CD3-PacificBlue; clone 500A2	BD Biosciences, San Jose, CA, USA
Anti-mouse-CD4 for CD4 cell depletion experiments; clone GK1.5	Harlan laboratories, Indianapolis, IN, USA
Anti-mouse-CD8-PE/Cy7 for FACS staining; clone Ly-2	BD Biosciences, San Jose, CA, USA
Anti-mouse-CD8 for CD8 cell depletion experiments; clone 2.34	Harlan laboratories, Indianapolis, IN, USA
Anti-mouse-IgG-POD	Amersham Biosciences Europe GmbH, Freiburg

ANTIBODIES	VENDOR
Biotin-labeled IFN-γ antibody; clone XMG1.2	BD Biosciences, San Jose, CA, USA
ELISpot IFN-γ capture antibody; clone R4-6A2	BD Biosciences, San Jose, CA, USA
FcγIII/II Receptor; clone 2.4G2	BD Biosciences, San Jose, CA, USA
Goat-anti-mouse-Cy3	Invitrogen, Carlsbad, CA, USA
NS3 antiserum from hybridoma cell culture („#4“)	Corapi et al., 1990
PE-labeled rat-anti-mouse IgG $F(ab)_2$	Invitrogen, Carlsbad, CA, USA

Table 3.5: Kits

KIT	VENDOR
ACK lysis buffer	Quality Biological, Inc., Gaithersburg, MD, USA
AP conjugate substrate Kit	Biorad, Hercules, CA, USA
BD Cytofix/Cytoperm™	BD Biosciences, San Jose, CA, USA
Bradford assay reagents	Pierce, Rockford, IL, USA
$CD8a^+$ T Cell Isolation Kit, mouse	Miltenyi, Bergisch Gladbach, Germany
ECL solution (western blot detection reagent)	Pierce, Rockford, IL, USA
FLAG© Tagged Protein Immunoprecipitation Kit	Sigma-Aldrich, St. Louis, MO, USA
Gel extraction kit	Qiagen, Valencia, CA, USA
LIVE⁄DEAD® Fixable Violet Dead Cell Stain Kit	Invitrogen, Carlsbad, CA, USA
Qiafilter Maxiprep kit	Qiagen, Valencia, CA, USA
Qiafilter or HiSpeed Midiprep kit	Qiagen, Valencia, CA, USA
RNeasy kit	Qiagen, Valencia, CA, USA
tip100©-columns	Qiagen, Valencia, CA, USA
VenorGeM® Mycoplasma Detection Kit	Sigma-Aldrich, St. Louis, MO, USA

Table 3.6: Additional materials

MATERIALS	VENDOR
Capillaries, heparinized	Drummond Scientific, Broomall, PA, USA
Caliper	VWR, West Chester, PA, USA
Cell strainer; 40 µm	BD Biosciences, San Jose, CA, USA
MultiScreenHTS plates (for ELISpot)	Millipore, Billerica, MA, USA
Mycokill	PAA, Pasching, Austria
Nitrocellulose membrane	Millipore, Billerica, MA, USA

Table 3.7: List of equipment and accessories

TYPE	VENDOR
AID EliSpot READER (VERSION 3.5)(Build 2550)	Autoimmun Diagnostika GmbH, Straßberg, Germany
AutoMACS™ Separator	Miltenyi, Bergisch Gladbach, Germany
FACSCalibur with software CellquestPro	Beckton-Dickinson, Heidelberg, Germany
Fluorescence microscope Axiovert 100 M with HBo-lamp and filters	Carl Zeiss, Jena, Germany
GenePulserII	Biorad, Hercules, CA, USA
LSRII flow cytometer	BD Biosciences, San Jose, CA, USA
Phosphor-Imager	GE Healthcare, Buckinghamshire, England

Table 3.8: Bacteria strains

NAME	APPLICATION
E.*coli* HB101 genetics	Used for all cloning steps and for amplification of constructs containing parts of the BVDV replicon
E.*coli* top 10	Used for cloning steps 1 and 2

Table 3.9: Plasmids

PLASMID	DESCRIPTION	SOURCE
DI9c	Monocistronic cytopathic DI9cBVDV replicon (Behrens et al., 1998)	Dept. Microbial Biotechnology, MLU
bi-cp-vector (H502)	Template plasmid for transcription of a bicistronic, cytopathic replicons encoding for beta-glucuronidase (Figure 3.1)	Dept. Microbial Biotechnology
pBluescipt KS^+	Cloning vector	Dept. Microbial Biotechnology
pORF-mIL-12	Source of IL-12 gene serving as template for the p35- and p40 subunit combined by a polypeptide linker	InvivoGen, San Diego, CA, USA
pUC18	Cloning vector	Dept. Microbial Biotechnology
FsphHer2-H502 (S12)	Template plasmid for transcription of a bicistronic, cytopathic replicons encoding for human Her2 (Figure 3.1)	Dept. Microbial Biotechnology
MpSP64-H506 (S17)	Construct containing the 5' sequence of the BVDV replicon (Figure 3.1)	Dept. Microbial Biotechnology
*r*Her2 plasmid	Plasmid encoding rat Her2	Louis Weiner, Fox Chase Cancer Center, Philadelphia, PA, USA

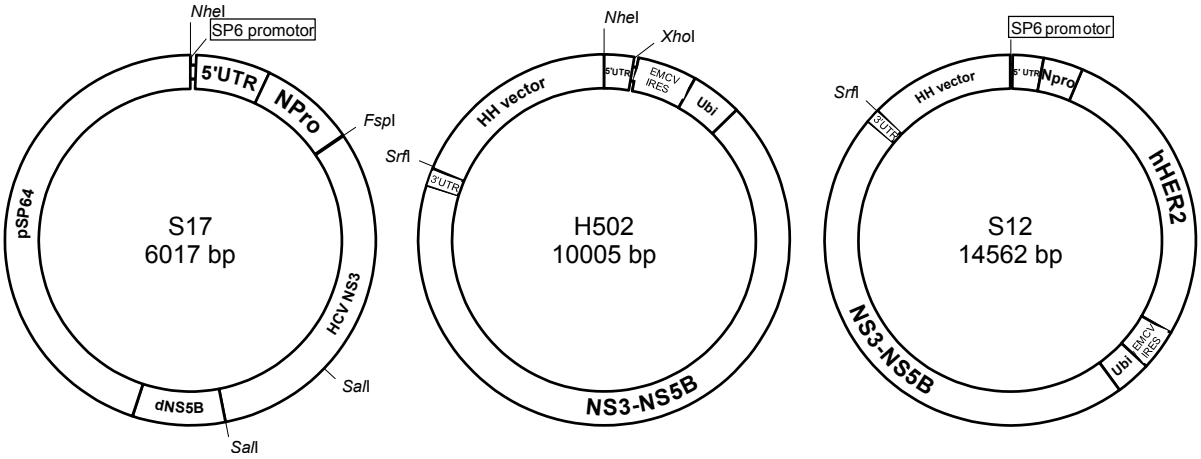

Figure 3.1: Organization of plasmids

Vector maps depict coding sequences and original plasmids.
Relevant restriction sites are indicated by the respective enzyme name. All plasmids contain the Ampicillin resistance gene.
5' UTR/3' UTR: coding sequence for the 5'/3' untranslated region of BVDV; NPRO, NS3-NS5B: coding sequence for the non-structural proteins of BVDV; dNS5B: part of the sequence encoding NS5B; HCV NS3: sequence encoding the non-structural protein 3 of the hepatitis C virus; pSP64: part of the sequence of the pSP64 poly(A) vector (Promega, Madison WI, USA), FspI restriction site was deleted by site-directed mutagenesis; HH: sequence from the monocistronic replicon DI9c described by Behrens et al., 1998; EMCV IRES: sequence encoding the Encephalomyocarditis virus internal ribosome entry site. Ubi: ubiquitin gene; hHer2: human Her2 gene.

Table 3.10: List of cloned constructs

Cloning steps are explained at 4.1.1 Construction of replicons.

INSERT	CLONING STEP	NAME	LINEARIZATION
*h*Her2-ECD	1	pBSK-Flag1	
	2	pBSK-*h*Her2-ECD-Flag	
	3	S17- *h*Her2-ECD	
	4	H502- *h*Her2-ECD	*Sma*I
*h*Her2-MF	1	pBSK-Flag2	
	2	pBSK-*h*Her2-MF-Flag	
	3	S17- *h*Her2-MF	
	4	H502- *h*Her2-MF	*Srf*I
*h*Her2-ICD	1	pBSK-Flag3	
	2	pBSK-*h*Her2-ICD-Flag	
	3	S17- *h*Her2-ICD	
	4	H502- *h*Her2-ICD	*Srf*I
*r*Her2-ECD	1	pBSK-Flag4	
	2	pBSK-*r*Her2-ECD-Flag	

INSERT	CLONING STEP	NAME	LINEARIZATION
	3	S17- *r*Her2-ECD	
	4	H502- *r*Her2-ECD	*Srf*I
*r*Her2-MF	1	pBSK-Flag5	
	2	pBSK-*r*Her2-MF-Flag	
	3	S17- *r*Her2-MF	
	4	H502- *r*Her2-MF	*Srf*I
*r*Her2-ICD	1	pBSK-Flag6	
	2	pBSK-*r*Her2-ICD-Flag	
	3	S17- *r*Her2-ICD	
	4	H502- *r*Her2-ICD	*Srf*I
*m*IL12	1	pBSK-Flag7	
	2	pBSK-*m*IL12-Flag	
	3	S17- *m*IL12	
	4	H502- *m*IL12	*Sma*I

Table 3.11: Materials used for cultivating eukaryotic cells

DESCRIPTION	VENDOR
β-mercaptoethanol, 55 mM in PBS	GIBCO, Carlsbad, CA, USA
DMEM high glucose	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA
Fetal bovine serum(FBS), heat inactivated	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1 M	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA
HL-1 (serum-free medium)	BioWhittaker (Lonza)
Horse serum	GIBCO, Carlsbad, CA, USA
L-glutamine, 200 mM	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA
Non-Essential Amino Acids (NEAA), 100x	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA
Penicillin, 10,000 U/m; Streptomycin, 10 mg/ml	GIBCO, Carlsbad, CA, USA; Cellgro, Manassas, VA, USA
RPMI-1640 without glutamine	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA
Sodium pyruvate, 100 mM	Cellgro, Manassas, VA, USA
Trypsin-EDTA 0.25 % trypsin, 2.21 mM EDTA in HBSS	Lonza, Cologne, Germany; Cellgro, Manassas, VA, USA

Table 3.12: Cell lines

NAME	ORGAN-ISM	CELL TYPE, ORGAN	MEDIA
2.43	mouse/ rat	hybridoma expressing CD8 antibodies	DMEM; 10% FCS; 1% penicil-lin/streptomycin
3T3/ neu	mouse	NIH3T3 expressing *r*Her2	DMEM; 10% FCS; 1% penicil-lin/streptomycin; 300 nM methotrexate
DC2.4	mouse	Immature DCs	RPMI; 10% FCS; 1% penicillin/streptomycin; 1% 1M HEPES pH 7,4; 1% NEAA; 2 mM L-glutamine; 55 µM β-mercaptoethanol
GK1.5	mouse/ rat	Hybridoma expressing CD4 antibodies	IMEM, 20% FCS, 1% penicillin/streptomycin
MDBK	bovine	kidney	DMEM; 5% FCS; 1% penicillin/streptomycin; hypoxanthine, d-biotin
NIH 3T3	mouse	fibroblasts	DMEM; 10% FCS; 1% penicil-lin/streptomycin
NT-2	mouse	*r*Her2-expressing tumor	RPMI; 20% FCS; 1% penicillin/streptomycin; 1% 1M HEPES pH 7,4; 1% NEAA; 2 mM L-glutamine; 1% Na-pyruvate; 100 mU/ml insulin

Table 3.13: Transfection systems

TRANSFECTION REAGENT	VENDOR
fuGENE HD	Roche Applied Sciences, Indianapolis, IN, USA
jetPEI	Polyplus transfection, Illkirch, France
TransIT®-mRNA Transfection Kit	Mirus, Madison, WI, USA
TransMessanger	Qiagen, Valencia, CA, USA

Table 3.14: List of software

NAME	COMPANY	APPLICATION
FlowJo 8.8.1	Tree Star, Inc., Ashland, OR, USA	Analysis of FACS data
Graph Pad Prism 5.0a	GraphPad Software, Inc., La Jolla, CA, USA	Data graphing and analysis
BD FACS Diva Soft-ware, version 5.0.3	BD Biosciences, San Jose, CA, USA	Aquisition of FACS data with the LSRII flow cytometer
Scion Image	Scion, Frederick, MD, USA	Density measurement for west-ern blot images

3.2 Methods

3.2.1 Molecular biology

Amplification of plasmids

Preparation of chemo-competent bacteria cells

Competent cells were prepared using the $RbCl_2$ method (Hanahan, 1983). A single colony of E.*coli* was used to inoculate 250 ml LB medium. Subsequently, cells were grown over night, pelleted at 5000 g at 4 °C for 5 min, resuspended in 100 ml transformation buffer I and incubated on ice for 5 min. Centrifugation was repeated and cells were resuspended in transformation buffer II. Following a 15–60 min incubation time, E.*coli* cells were aliquoted, shock frozen in liquid nitrogen and stored at -80 °C.

Transformation

Plasmid DNA was transformed into chemo-competent E.*coli* cells following the heat shock procedure. To this end, 30 µl chemo-competent cells were incubated with 1 µg plasmid DNA or the complete ligation reaction on ice for 20 min, shocked for 90 s at 42 °C, incubated for 2 min on ice and finally incubated at 37 °C in a thermomixer. Afterwards, cells were plated on an LB-agar plate containing the respective antibiotics for selection. Transformed cells were grown at 37 °C overnight.

E.coli cultures

LB-Medium was inoculated with a single transformation colony. E.*coli* cells were grown overnight in an incubation shaker at 37 °C in the presence of antibiotics for selection.

Cloning methods

Isolation of DNA

DNA was isolated from E.*coli* cells in small, medium or large scale following the principle of alkaline lysis (Birnboim & Doly, 1979).
A culture volume of 2 ml was inoculated for a small-scale isolation and DNA was purified by ethanol precipitation. 50–100 ml were inoculated for a medium-scale preparation and DNA was purified using tip100©-columns (Qiagen) or a Qiagen Midiprep kit. For large-scale production bacteria were grown in 400 ml medium and DNA was isolated and purified using the Qiafilter Maxiprep kit (Qiagen) according to the manufacturer's protocol.

Restriction digest

DNA was digested with two units of restriction enzyme per microgram DNA at the temperature and in the buffer specified by the manufacturer for the enzyme or enzyme combination for at least 2 h. Preparative digestions were incubated overnight and the restriction fragments were isolated from an agarose gel using a gel extraction kit (Qiagen) according to the manufacturer's protocol.

Ligation

DNA fragments were linked in a ligation reaction using 500 ng digested plasmid and a five-fold molar excess of the insert DNA in the presence of 1U T4 DNA ligase in a total volume of 10–20 µl overnight at 16 °C. The complete ligation reaction was transformed.

Preparation of helper oligonucleotides

For each construct two single stranded oligonucleotides were synthesized containing a complementary sequence of 15–17 bases. Both oligonucleotides were mixed at an equimolar ratio, incubated for 5 min at 75 °C to disrupt intramolecular base-pairing and cooled down slowly to room temperature to specifically hybridize the single strands.
The remaining single stranded regions were filled up in a Klenow reaction in the presence of deoxyribonucleotides at 37 °C for 10 minutes. The enzyme was inactivated by adding 2 mM EDTA and incubating the reaction mix at 70 °C for 15 min. The resulting double stranded DNA was further used in a restriction digestion after titration of EDTA with 4 mM magnesium ions.

Cloning of replicon constructs

A detailed description of the cloning procedure of replicon constructs can be found in the results chapter 4.1.1.
Restriction enzymes used for the individual clones in step 2 are listed in Table 3.15.

Table 3.15: List of cloning vectors and restriction enzymes used to clone the individual constructs

		RE1		RE2	
INSERT	CLONING VECTOR	A	B	A	B
*h*Her2-ECD	pBluescipt KS$^+$	*Xho*I	*Not*I	*Stu*I	*Aat*II
*h*Her2-MF	pUC18	*Sac*I	*Sal*I	*Bgl*II	*Kpn*I
*h*Her2-ICD	pBluescipt KS$^+$	*Xho*I	*Xho*I	*Aat*II	*Nde*I
*r*Her2-ECD	pBluescipt KS$^+$	*Xho*I	*Xho*I	*Aat*II	*Nde*I
*r*Her2-MF	pBluescipt KS$^+$	*Xho*I	*Xho*I	*Bgl*II	*Nco*I
*r*Her2-ICD	pBluescipt KS$^+$	*Xho*I	*Xho*I	*Nde*I	*Stu*I
*m*IL-12	pBluescipt KS$^+$	*Xho*I	*Not*I	*Nco*I	*Avr*II

In-vitro-transcription

In-vitro-transcription was used to produce replicon-RNA of linearized template DNA. A transcription reaction typically contained the following reagents:

transcription-buffer	10x	10	µl
rNTP-mix	10 mM	10	µl
DTT	100 mM	10	µl
RNase inhibitor	100 mM	1	µl
RNA polymerase (SP6)	20 U/ml	2.5	µl
DNA template, linearized		2	µg
ad. H_2O		100	µl

The reaction mix was incubated at 37 °C for 3 h and another 30 min after the addition of 250 U DNase I (Roche) to digest the template DNA. The transcripts were purified either by phenol-chloroform-extraction followed by ammonium-acetate-precipitation or with the RNeasy kit (Qiagen) according to the manufacturer's protocol.
The concentration was determined by UV absorption of the RNA solution and the quality was analyzed on an agarose-formamide gel. RNA was aliquoted and stored at -40 to -80 °C.

In-vitro-translation

In-vitro-translation was used to analyze antigen encoding by the replicons. A translation reaction typically contained the following reagents:

ATP	100	mM	0.25	µl
GTP	10	mM	0.5	µl
potassium acetate	5	M	0.6	µl
magnesium acetate	100	mM	0.65	µl
HEPES buffer	1	M	0.75	µl
DTT	100	mM	0.75	µl
creatine phosphate	0,5	M	2.5	µl
creatine phosphate kinase	10	mg/ml	2	µl
RNase inhibitor	40	U/µl	0.5	µl
S10-Extrakt from Huh7 cells			7.5	µl
Promix 35Met/35Cys			0.7	µl
template RNA			1	µg
ad. H_2O			25	µl

The reaction mix was incubated at 30 °C for 4 h, mixed with 8 µl protein sample buffer and incubated at 95 °C for 5 min. Proteins in the mix were separated on a 12% polyacrylamide gel and radioactively labeled proteins were visualized by exposing the dried gel to a phosphorimager screen for 12–36 hours and reading the screen on a phosphorimager (GE Healthcare).

3.2.2 Cell biology

Transfection of RNA replicons by electroporation

Cells were split 16–24 hours prior to transfection to achieve 80% confluency at the time of harvest. MDBK or DC2.4 cells were trypsinized, resuspended in the respective medium, washed twice and resuspended in PBS. The cell suspension was mixed with RNA, transferred in an electroporation cuvette and pulsed at the indicated parameters (see table Table 3.16). Subsequently, cells were transferred from the cuvette to a cell culture plate in two times 800 µl medium. Transfection efficiency was determined 16–28 h post transfection by immunohistochemistry or flow cytometry.

Table 3.16: Electroporation conditions for different cell types

CELL LINE	NO. OF CELLS	RNA	CUVETTE	VOLUME	VOLTS	µFARADS	OHMS
MDBK	ca. $4x10^6$	3 µg	2 mm	400 µl	180	950	∞
DC2.4	$2x10^6$	5 µg	4 mm	200 µl	300	750	400

Subcloning of DC2.4 cells by limiting dilution

To isolate potential subpopulations of DC2.4 cells that exhibited higher transfection efficiencies, cells were subcloned by limiting dilution. A cell suspension was diluted to 50, 1.25 and 0.3125 cells/ml and cells were plated in a 96-well-plate for each dilution with 200 µl per well to have an average of 10, 2.5 and 0.8 cells per well, respectively. After one week, colonies derived from a single cell were visible. Wells with one colony only were identified and cells from these wells were transferred to a 24-well-plate and, after growing confluent, to a 6-well-plate and tested for transfection efficiency.

Immunohistochemistry

Immunofluorescence staining of cells fixed on cover slips was used to identify replicon-transfected cells.

Transfected cells were grown on sterile cover slips. To prepare staining of the intracellularly produced BVDV-NS3 protein, cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, at room temperature for 15 min. Subsequently, cells were washed twice with PBS for 5 min and permeabilized with 0.5% N-octylglycopyranoside in PBS, pH 7.4, at 4 °C for 10 min. After washing the cells twice again with PBS for 2 min, blocking-solution (5% FCS in PBS, pH 7.4, 0.05% Tween20) was added for 10–15 min at room temperature. To stain the cells they were first incubated with a 1:10 dilution of the primary mouse anti-BVDV NS3 antibody (#4) at 37 °C for 1 h, washed thrice with PBS, 0.05% Tween20, pH 7.4, at room temperature for 2 min and then incubated with the secondary, Cy3-labeled, goat-anti-mouse antibody (1:200). After additional

three washing steps the cover slip was inverted with the cells facing down in a drop of PBS onto a microscope slide.
Transfected cells were visualized by detecting the fluorescent light emitted by the Cy3 molecule attached to the secondary antibody. Transfection efficiency was determined by the ratio of transfected to untransfected cells counted on an image section containing 100–500 cells.

Flow cytometry

Flow Cytometry was used to analyze (i) depletion of T cells in mice as well as (ii) transfection efficiency and (iii) cell death in replicon-transfected cells. The LSRII flow cytometer (BD Biosciences) was used for most applications.
Washing steps were performed by adding 2 ml FACS buffer and centrifuging at 340 g for 7 min.

Test for depletion of T lymphocyte subsets by surface staining

Splenocytes from depleted mice were washed once with FACS buffer, resuspended in 100 µl and incubated with 5 µl ethidium monoazide on ice under a 40 W fluorescent light for 10 min to stain for dead cells. Cells were washed once, resuspended in 100 µl FACS buffer and incubated with the surface antibodies, 3 µl CD3-PacificBlue, 1 µl B220-PE-Cy5, 0.2 µl CD8-PE-Cy7 and 0.5 µl CD4-APC-Cy7, at 4 °C for 20 min. After two washing steps cells were fixed in 150 µl 1% paraformaldehyde in PBS.

Intracellular staining of transfected cells

Transfected cells were washed once in FACS buffer, resuspended in BD Cytofix/Cytoperm™ permeabilization solution and incubated at 4 °C for 20 min. After washing the sample twice with BD Cytofix/Cytoperm™ wash solution the cells were resuspended in 50 µl and incubated with 2 µl of the primary antibody, mouse anti-BVDV NS3 antibody (#4), at 4 °C for 20 min. Unbound antibody was removed by two additional washing steps and 50 µl of the cell suspension was incubated with 2 µl secondary antibody, PE-labeled rat-anti-mouse IgG $F(ab)_2$ fragment, at 4 °C for 20 min. Subsequently, cells were washed twice and fixed in 150 µl 1% paraformaldehyde.

Dead cell staining

Cells were trypsinized and pooled with the cell culture supernatant to collect dead cells that have detached from the culture dish. After one washing step the cells were resuspended in 200 µl FACS buffer. Dead cells were stained by adding 100 µl propidium iodide (2.5 µg/ml in PBS) and incubating the cell suspension at room temperature in the dark for 15 min. Cells were analyzed by flow cytometry with the FACScalibur.

Determining the time course of the cytopathic effect

Cell death after replicon transfection was analyzed over time by FACS analysis of cells stained for cell death and NS3 expression.

For this experiment, cells from three transfections with Repl-rMF were pooled and then distributed equally in a 6-well-plate. The samples were obtained 15 h, 35 h and 51 h post transfection by collecting the cell culture supernatant and the trypsinized cells. Each sample was stained immediately for dead cells by incubating the PBS-washed cells in 100 µl PBS containing 1 µl of LIVE/DEAD® Fixable Violet Dead Cell Stain (Invitrogen) diluted 1:10 at room temperature for 30 min. Cells were washed in PBS and then fixed in 4% paraformaldehyde at 4 °C for 10 min, washed twice with FACS buffer and stored at 4 °C until all samples were collected.

Subsequently, cells were permeabilized (see "Intracellular staining" under 3.2.2) and indirectly stained for NS3 using 2 µl of the mouse-anti-NS3 antibody (#4) and 2 µl of PE-labeled rat-anti-mouse IgG $F(ab)_2$ fragment. NS3-expression was analyzed with the PE filter set and violet dead cell stain was detected in the PacificBlue channel of the LSRII flow cytometer (BD Biosciences).

Preparation of overlapping peptide (OLP) pool mixes

OLP pools were composed of 20mer peptides overlapping by ten amino acids that covered the sequence of the extracellular domain (ECD) of Her2. Peptide pools were synthesized at a purity of >50% and a scale of 1–3 mg per peptide (Mimotopes, Australia).

Each of the 44 peptides (Table 3.17) was dissolved in 50 µl DMSO and diluted in 950 µl PBS. Peptides 1–22 and 23–44 were mixed and named OLP-mix I and OLP-mix II, respectively. Stock solutions were stored at -20 °C and working solutions were kept at 4 °C.

Table 3.17: Peptides in overlapping peptide pools

NAME	SEQUENCE	NAME	SEQUENCE
#1	DVFRKNNQLAPVDIDTNRSR	#23	PEQLQVFETLEEITGYLYIS
#2	PVDIDTNRSRACPPCAPACK	#24	EEITGYLYISAWPDSLRDLS
#3	ACPPCAPACKDNHCWGESPE	#25	AWPDSLRDLSVFQNLRIIRG
#4	DNHCWGESPEDCQILTGTIC	#26	VFQNLRIIRGRILHDGAYSL
#5	DCQILTGTICTSGCACGRLP	#27	RILHDGAYSLTLQGLGIHSL
#6	TSGCACGRLPTDCCHEQCAA	#28	TLQGLGIHSLGLRSLRELGS
#7	TDCCHEQCAAGCTGPKHSDC	#29	GLRSLRELGSGLALIHRNAH
#8	GCTGPKHSDCLACLHFNHSG	#30	GLALIHRNAHLCFVHTVPWD
#9	LACLHFNHSGICELHCPALV	#31	LCFVHTVPWDQLFRNPHQAL
#10	ICELHCPALVTYNTDTFESM	#32	QLFRNPHQALLHSGNRPEED
#11	TYNTDTFESMHNPGRYTFGA	#33	LHSGNRPEEDCGLEGLVCNS
#12	HNPGRYTFGASCVTTCPYNY	#34	CGLEGLVCNSLCAHGHCWGP
#13	SCVTTCPYNYLSTEVGSCTL	#35	LCAHGHCWGPGPTQCVNCSH

NAME	SEQUENCE	NAME	SEQUENCE
#14	LSTEVGSCTLVCPPNNQEVT	#36	GPTQCVNCSHFLRGQECVEE
#15	VCPPNNQEVTAEDGTQRCEK	#37	FLRGQECVEECRVWKGLPRE
#16	AEDGTQRCEKCSKPCARVCY	#38	CRVWKGLPREYVSDKRCLPC
#17	CSKPCARVCYGLGMEHLRGA	#39	YVSDKRCLPCHPECQPQNSS
#18	GLGMEHLRGARAITSDNVQE	#40	HPECQPQNSSETCFGSEADQ
#19	RAITSDNVQEFDGCKKIFGS	#41	ETCFGSEADQCAACAHYKDS
#20	FDGCKKIFGSLAFLPESFDG	#42	CAACAHYKDSSSCVARCPSG
#21	LAFLPESFDGDPSSGIAPLR	#43	SSCVARCPSGVKPDLSYMPI
#22	DPSSGIAPLRPEQLQVFETL	#44	VKPDLSYMPIWKYPDEEGIC

Enzyme-linked immunosorbent spot (ELISpot) assay

The ELISpot assay was used to determine the frequency of IFN-γ producing Her2-specific CD8+ T cells induced by vaccination with replicon-transfected DC2.4 cells. To this end, T cells were isolated from spleens of vaccinated mice and specifically stimulated with OLP-Mix II (referred to as OLP) or DMSO as a negative control. Irradiated NIH3T3 mouse fibroblasts (FVB mouse background; H-2^q) were used as APCs.
Coating of Elispot plates with IFN-γ capture antibody was achieved by adding 100 µl of 3 µg/ml antibody solution per well and incubating at 4 °C overnight. Wells were washed four times with 200 µl PBS and then blocked with RPMI containing 5% heat inactivated horse serum for 2–3 h. Following two washing steps with 200 µl PBS per well, plates were equilibrated with serum-free HL-1 medium for 30 min.

Isolation of CD8+ T cells from spleen

Vaccinated mice were sacrificed at the end of the tumor challenge experiment to obtain the spleen. Splenocytes were isolated by mechanical disruption of the organ with subsequent filtering of the cells through a 40 µm cell strainer. Erythrocytes were lysed by incubating the cells in 3 ml ACK lysis buffer for 2 min. Cells were washed with PBS, counted and then subjected to CD8+ T cell isolation.
CD8+ T cells were separated using the negative isolation kit in combination with the autoMACS™ Separator (Miltenyi) according to the manufacturer's protocol. This method applies magnetic beads conjugated to antibodies specific for cell surface proteins. Antibody-labeled cells are separated from unlabeled cells by a magnet. Negative separation of cells is facilitated by labeling all cells included in a splenocyte suspension except CD8+ cells by adding antibodies specific for CD4+ T cells, monocytes/macrophages, B cells, natural killer cells and erythrocytes (targeted antigens: CD4, CD11b, CD45R, DX5, Ter-119). The purity of CD8+ T cells in the unlabeled fraction was 90% as confirmed by FACS analysis.

ELISpot assay

All components added to the wells were resuspended or diluted in serum-free HL-1 medium. NIH3T3 ("3T3wt") cells, serving as APCs, were irradiated at 10,000 rad, re-

suspended at 1x10^6 cells/ml and added in 50 µl to each well of the emptied ELISpot plate. 8 µl OLP-Mix II or 5% DMSO in PBS were added in another 50 µl to the wells indicated. Serial dilutions of CD8+ T cells were prepared in a 96-well-plate and the cells were transferred in 100 µl per well to the ELISpot plate.
Plates were incubated at 37 °C and 5% CO_2 for 36–48 h, washed three times with PBS and then four times with PBS/Tween (1:2000). The secondary, biotin-labeled IFN-γ antibody was added in 100 µl PBS/Tween/1% BSA per well and plates were incubated in a humidified box at 4 °C for 12–24 hours. Plates were washed four times with PBS/Tween from both sides and Streptavidin was added in a 1:2000 dilution in PBS/Tween/BSA in 100 µl per well. Plates were incubated at room temperature for 1 h and then developed with the AP conjugate substrate kit (Biorad) according to the manufacturer's protocol. Finally, plates were washed extensively with water, dried and analyzed with an ELISpot plate reader (Autoimmun Diagnostika GmbH).
The number of Her2-specific cells was determined by subtracting SFU from control stimulation with PBS+DMSO from SFU counted for stimulation with the OLP-Mix II. To account for inter-assay variability, the spleen cells of one of the mock-vaccinated mice (mouse M3) were tested in each assay to normalize SFU by the variation factor. Consequently, SFU counted for mice M5-M8 of the ECD group and mice M5-M8 of the IL-12 group were multiplied by 2.2 and mice M5-M7 of the MF group as well as mice M5-M7 of the MF+IL-12 group were multiplied by 4.3.

Antibody assay

Production of Her2-specific antibodies in response to vaccination was analyzed by a modified protocol from Reilly et al. (2000). Briefly, blood was collected by retroorbital bleeding ten days after the second vaccination and plasma was isolated by centrifugation of the heparinized capillaries at 10,000 g for 10 min. The undiluted plasma was incubated with 2x10^5 3T3wt or with Her2-expressing 3T3 cells (3T3/neu), respectively. Antibodies bound to the cells were detected by staining with a PE-labeled anti-mouse-IgG antibody followed by flow cytometry. Anti *r*Her2 antibody was used as positive control (3.3 ng/ml).

3.2.3 Protein biochemistry

Immunoprecipitation

In order to confirm the expression of the heterologous protein encoded by the replicon constructs, the lysates of transfected MDBK cells were analyzed for the FLAG-tagged proteins by western blotting. Since the detection of some of the proteins turned out to be difficult, proteins were concentrated from the cell lysate by immunoprecipitation prior to western blotting.

Cell lysis

Cells from six transfections were harvested 24 h post transfection, washed twice in ice-cold PBS and resuspended in 1 ml cell lysis buffer including protease inhibitor mix (Roche) and incubated at 8 °C for 30 min. After centrifugation at 13,000 g at 4 °C for 10 min to pellet the cell debris, the supernatant was transferred to a new tube.

FLAG immunoprecipitation

First, the anti FLAG M2-Agarose Affinity Gel (Sigma) was prepared for immunoprecipitation: 10 µl of affinity gel were centrifuged at 5000 g for 10 min, washed twice with wash buffer, incubated with elution buffer for 5 min on ice and washed thrice in wash buffer.
Second, affinity gel was added to the cell lysate and incubated at 4 °C for 4 h. The resin was washed thrice in wash buffer and bound protein was eluted with 50 µl elution buffer by incubation at room temperature for 5 min. After centrifugation the supernatant was collected and 5 µl 10x concentrated wash buffer were added.

Western Blot

Proteins from cell lysate or eluate of an immunoprecipitation were separated on an SDS polyacrylamide gel and transferred to a nitrocellulose membrane by semi-dry-blotting (Kyhse-Anderson, 1984). The transfer was established at 30 V and 0.8 mA/cm² and 2.5 h. The nitrocellulose membrane was blocked in TBS containing 1% milk powder at 4 °C overnight, incubated with a 1:1000 dilution (in 1% milk powder solution) of anti-Flag M2 antibody for 1 h, washed thrice for 10 min in TBT buffer and incubated for 1h in the secondary antibody solution, horse radish peroxidase coupled anti-mouse-IgG-antibody 1:7500 (in 1% milk powder solution in TBS), and washed again three times in TBT buffer. Incubation and washing steps were performed at room temperature.
The membrane was treated with detection solution for 5 min and then exposed to an X-ray film.

Western blot to compare relative replication efficiency

Replication efficiency of the replicons was compared by determining the relative amount of NS5A in transfected cells using a western blot assay. MDBK cells were transfected in triplicate with each replicon. Cells from one transfection were collected in a 10-cm-dish and harvested 24 h post transfection by scraping the cells off the plate. Protein concentration of the cell lysate (1:10) was determined by the Bradford method (Bradford, 1976) and the volume containing 200 µg was loaded on a 10% polyacrylamide gel.
The western blot was carried out as described above. The nitrocellulose membrane was cut between the marker band 55 kDa and 75 kDa and the upper part, containing

the slower migrating proteins, was incubated in a 1:500 dilution of NS5A antiserum from rabbit whereas the other part was incubated in a 1:5000 dilution of mouse anti Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody. The respective secondary detection reagent was used at a 1:7500 dilution each.
Relative expression quantitation was achieved by determining the signal density with the software Scion Image.

IL-12 ELISA (Enzyme-linked immunosorbent assay)

DC2.4 cells were transfected with Repl-IL12 in triplicate and Repl-rMF, resuspended in 3 ml medium and plated in one well of a 6-well plate for each transfection. The supernatant of transfected cells was collected at 14 h, 24 h and 48 h post 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 of the centrifugation was diluted four-fold and subjected to an ELISA specific for the bioactive form of murine IL-12 (eBio-sciences). The assay was performed according to the manufacturer's protocol.

3.2.4 Mouse experiments

All mouse experiments were performed with H-2^q FVB/N mice (Taketo et al., 1991; Jackson Laboratory, Bar Harbor, ME) under a protocol approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases.

Vaccination and tumor challenge

DC2.4 cells were transfected 14 h prior to injection into mice. Cells were trypsinized, resuspended in medium, washed twice and resuspended in PBS at a concentration of $5x10^6$ cells/ml. For vaccination with DC2.4 cells transfected with two replicons (Her2-replicon + Repl-IL12) the individually transfected cells were combined at a 1:1 ratio. All cells were kept on ice until injection.
Each mouse received a subcutaneous (s.c.) injection at the base of the tail of $1x10^6$ cells in 200 µl for individually transfected replicons and $2x10^6$ cells in 400 µl for vaccinations with combined replicons. Control groups were vaccinated with mock-transfected DC2.4 cells (electroporation in the absence of RNA).
Tumor challenge was performed by s.c. injection of $1x10^6$ NT-2 cells in the right flank. The cells were washed twice and resuspended in PBS prior to injection.

Preventive vaccination

Mice received the first injection at the age of 8–10 weeks followed by the second vaccination 3–5 weeks later. Two weeks after the last vaccination they were challenged with $1x10^6$ NT-2 tumor cells.

Therapeutic vaccination

Mice were first challenged with 1x10^6 NT-2 tumor cells. The first injection of replicon-transfected DCs was given when established tumors were visible, which was on day 5 post tumor challenge. Vaccinations were repeated every 7 days for a total of 4 times.

Measuring tumor size

Tumor size was determined thrice weekly starting on day 5 post tumor challenge. The largest (a) and smallest (b) dimension of the tumor was measured with a caliper to calculate the tumor volume (V) according to the following formula (Carlsson et al., 1982):

$$V = \frac{a \times b^2}{2}$$

T cell depletion

For depletion of T lymphocyte subsets, mice were injected intraperitoneally (i.p.) with 1 mg purified anti-CD4-antibody (clone GK1.5) or 1 mg purified anti-CD8-antibody (clone 2.43) on day -7, -2, 5, 12 and 19 of tumor challenge. Absence of the targeted cells at the time of tumor challenge was determined by FACS analysis of splenocytes from similarly treated mice. The depletion of CD4+ and CD8+ T cells in mice that were challenged with the tumor cells was confirmed at the end of the experiment on day 21 post tumor challenge. Depletion efficiency was determined by dividing the frequency of the T cell subset in depleted and undepleted mice.

Statistics

Statistical analysis was performed using the software Graph Pad Prism. All statistical analyses were performed as two-tailed tests. The Mann-Whitney U test was used to compare the tumor size on day 21 post tumor challenge between vaccinated mice and the mock control group or depleted and undepleted mice. Tumor growth prevention was analyzed with the Chi-square test. The Spearman test was used as a non-parametric analysis for correlation of tumor size and CD8+ T cell response. Abbreviations: ns – not significant; *– significant (P: 0.01–0.05); **– very significant (P: 0.001–0.01); ***– extremely significant (P<0.001).

4 Results

4.1 Generation of replicon-transfected dendritic cells as a tool for vaccination

4.1.1 Construction of replicons

The first task of this study was the construction of BVDV replicons that encoded fragments of the Her2 antigen and murine IL-12 (*m*IL-12), respectively. The heterologous genes were introduced in the first ORF of the bi-cistronic replicon (Figure 4.1) in frame with the BVDV N^{PRO} gene and a sequence encoding for the FLAG epitope (DYKDDDDK). Consequently, the heterologous proteins are translated in fusion with N^{PRO} and released by the autoprotease activity of N^{PRO} generating its own C-terminus (Wiskerchen et al., 1991). The FLAG epitope was introduced to the N-terminus of the heterologous protein to enable easy detection of proteins/protein fragments with a single antibody.
The second ORF of the replicon encodes the NS proteins of BVDV and expression is mediated by the EMCV IRES. The viral proteins NS3, NS4A, NS4B, NS5A and NS5B are needed to mediate the replication of the template RNA. The free NS3 determines the cytopathic nature of the replicon.

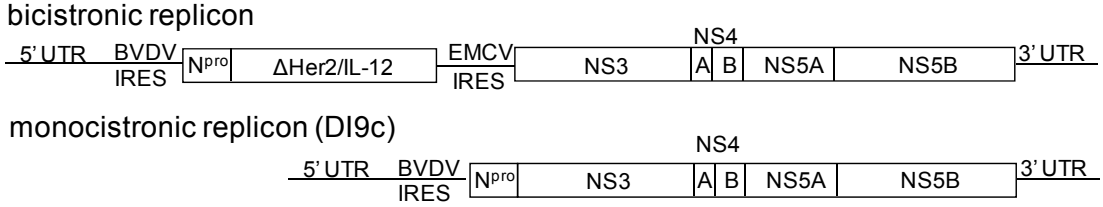

Figure 4.1: Schematic organization of the generated cytopathic, bi-cistronic BVDV replicon constructs in comparison to the mono-cistronic replicon DI9c. Her2 gene fragments or the IL-12 gene were cloned into the first ORF downstream of N^{PRO} and the FLAG sequence. UTR: untranslated region; FLAG: coding sequence for the FLAG epitope; ΔHer2: Her2-gene-fragments; IL-12: *m*IL-12 gene

In the case of the Her2 antigen (full-length human Her2: 3768 bases corresponding to 1256 aa, rat Her2: 3780 bases corresponding to 1260 aa), we decided to apply fragments of the gene since it was known that insertion of longer fragments into the BVDV replicon often resulted in inhibition of viral RNA replication. Hence, fragments of the rat and human Her2 gene (*h*Her2, *r*Her2) of about 2000 nucleotides in length were cloned into the replicons (Figure 4.2). The final constructs should encode the extracellular domain (*h*ECD: 403–1959 bases, 135–653 aa; *r*ECD: 523–1881 bases, 175–627 aa), a middle fragment (*h*MF: 1105–3078 bases, 369–1026 aa; *r*MF: 1117–

3015 bases, 373–1005 aa) and the intracellular domain (*h*ICD: 1951–3768 bases, 651–1256 aa; *r*ICD: 1873–3765 bases, 625–1255 aa) of Her2, respectively. The here-applied *r*Her2 fragments were shown to contain several MHC class I epitopes that are actively recognized in FVB/N mice (Singh et al., 2006; Singh & Paterson 2007, Ercolini et al., 2003).

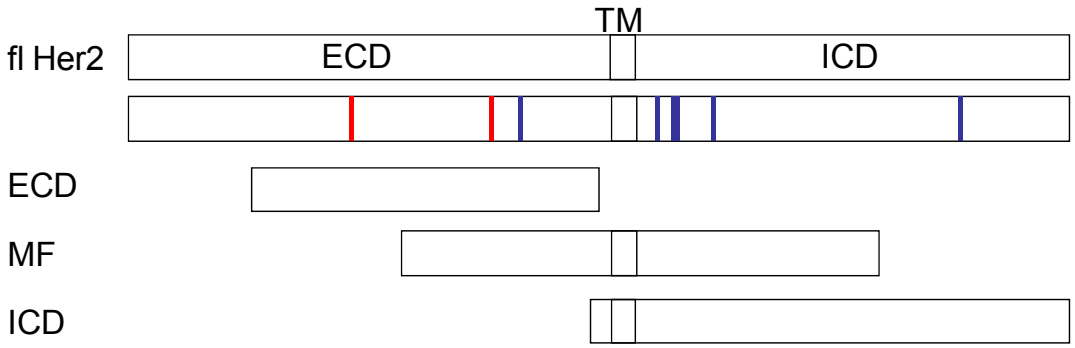

Figure 4.2: Epitope-rich fragments of the Her2 gene that were cloned into BVDV replicons

Full-length *r*Her2 (fl Her2) is depicted as a box containing three major domains, ECD (extracellular domain), TM (transmembrane) and ICD (intracellular domain). The positions of identified epitopes of *r*Her2 in FVB mice (Singh et al., 2006; Singh & Paterson 2007, Ercolini et al., 2003) are indicated in blue and red (immunodominant). The Her2 fragments, ECD, MF (middle fragment) and ICD, are depicted according to length and overlap with fl Her2.

Plasmid transcription templates for the bi-cistronic replicons encoding ECD, MF and ICD of *h*Her2 and *r*Her2 as well as *m*IL-12 were cloned in four steps:

1. Insertion of helper oligonucleotides into cloning vectors:
   For the cloning of each construct, a so-called “helper oligonucleotide” was synthesized, which contained several restriction sites and the FLAG coding sequence (Figure 4.3). Double-stranded versions of these oligonucleotides were cloned into the vectors pBluescript or pUC18 using the restriction sites RE1 a and b (see Methods; Table 3.15).

Figure 4.3: Schematic organization of applied helper oligonucleotides

Arrows indicate the site of heterologous gene insertion. RE1 and RE2: restriction sites required for cloning steps 1 and 2; FLAG: coding sequence of the FLAG epitope; Stop: translational stop codon; black: variable regions to facilitate cloning in translational reading frame; *Fsp*I, *Sal*I: restriction sites.

2. Cloning of the Her2 gene fragments and the *m*IL-12 gene downstream of the FLAG coding sequence:
   The Her2 gene fragments and the *m*IL-12 gene were excised from the respective template vectors (*h*Her2 fragments from FsphHer2-H502 (S12), *r*Her2 fragments from the *r*Her2 vector, IL-12 from pORF-mIL-12) using restriction sites corresponding to RE2 a and b (Figure 4.3). The fragments were then cloned into the predigested vectors derived from step 1. The resulting constructs encoded the heterologous proteins N-terminally fused with the FLAG epitope.
3. Establishing the sequence of the first replicon ORF:
   The heterologous genes with the FLAG sequence were cloned *Fsp*I/*Sal*I into the vector MpSP64-H506 (S17) (see 3.1 Materials). In this way, the sequences encoding the SP6 promoter, the BVDV 5'-UTR, the BVDV-IRES and the N^{PRO} gene were fused upstream to the FLAG encoding sequence, which together constitute the sequence encoding the first ORF of the replicon (Figure 4.4 A).
4. Establishing the plasmid serving as a template for the bi-cistronic replicons:
   The DNA fragments consisting of the BVDV 5' region and the Her2 gene fragments or the IL-12 gene were excised *Nhe*I/*Sal*I and cloned into the *Nhe*I/*Xho*I-digested bi-cp-vector (H502) (see 3.1 Materials). This vector encodes the second ORF of the bi-cistronic construct comprised of the BVDV non-structural proteins NS3–NS5B, the 3' UTR and a *Srf*I/*Sma*I restriction site for linearization (Figure 4.4 B).

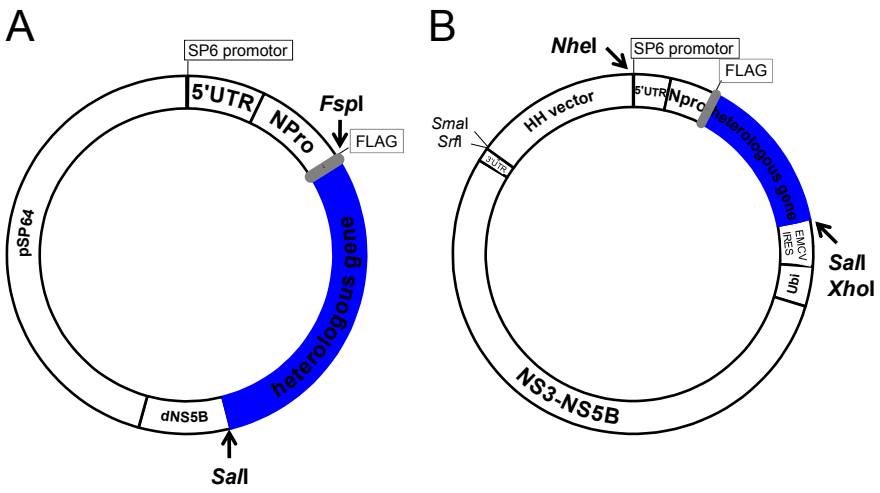

Figure 4.4: Organization of vectors for cloning of replicon template DNA

Arrows indicate the site of DNA fragment insertion labeled with the applied restriction enzyme. A: Resulting vector after the third cloning step. B: Final vector encoding the bicistronic replicon. The DNA encodes all genes included in the replicon RNA in addition to non-coding sequences, in particular the UTR and IRES regions, sequences required for transcription of the replicons (SP6 promotor) and a restriction site for linearization of the plasmid (*Srf*I/*Sma*I). Abbreviations: see Figure 3.1.

Correct cloning of the respective cDNA constructs was verified by *Pst*I digestion (Figure 4.5): i.e., for each of the generated plasmids, a restriction pattern was obtained where all bands had the expected, calculated sizes (Table 4.1).
The DNA template vectors were linearized with the restriction enzymes *Srf*I or *Sma*I (Figure 4.4, B) immediately downstream of the 3'UTR coding region and subsequently transcribed with the SP6 RNA polymerase to produce replicon RNA (Figure 4.6) (see 3.2.1 *In-vitro*-transcription). Resulting replicon RNAs were named Repl-hECD, Repl-hMF, Repl-hICD, Repl-rECD, Repl-rMF, Repl-rICD and Repl-IL12.

Heterologous protein	Vector-specific fragments (bp)
*h*ECD	350 + 1200 + 4600
*h*MF	430 + 800 + 5300
*h*ICD	450 + 1300 + 4700
*r*ECD	1400 + 4600
*r*MF	500 + 800 + 5300
*r*ICD	300 + 550 + 1200 + 4350
*m*IL-12	470 + 850 + 5000

Table 4.1: *Pst*I-digestion of replicon template vectors, calculated fragment sizes

The listed fragments are specific for the individual constructs while fragments of 250, 2500, 3300 base pairs (bp) are shared among all different vectors (not listed). *h*ECD, *h*MF, *h*ICD: extracellular fragment, middle fragment and intracellular fragment of *h*Her2; *r*ECD, *r*MF, *r*ICD: extracellular fragment, middle fragment and intracellular fragment of *r*Her2; *m*IL-12: murine IL-12.

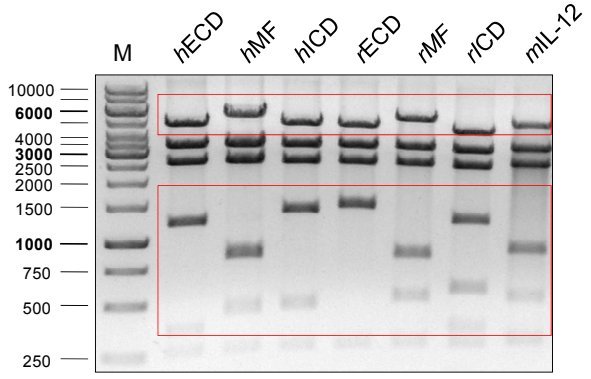

Figure 4.5: Restriction analysis of generated replicon cDNA constructs

Correct cloning was confirmed by *Pst*I-digestion of the DNA followed by the analysis of fragment sizes on a 1% TAE agarose gel. Plasmids are specified by the replicon insert. On the left of the gel the size of marker DNA (M) in bp is depicted. Red boxes frame construct-specific fragments.

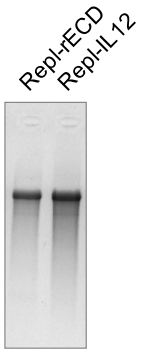

Figure 4.6: Production of RNA replicons by *in vitro* transcription of template DNA

As an example, 0.5 µg of RNA transcripts of Repl-rECD and Repl-IL12 template vectors were separated on a 1% denaturing formamide gel. As shown, the transcription products were pure and stable.

4.1.2 Transfection of DC2.4 cells

In this study we used the immature DC line DC2.4 (provided by K. Rock, Shen et al., 1997). DC2.4 cells have the potential capacity to mature, to process antigens and to upregulate MHC I, MHC II, CD80, CD86 and CD40 (Okada et al., 2001).
The transfection of RNA replicons is challenging in general, because the RNA molecules are large (about 12.5 kb) as compared to other RNAs such as siRNAs, miRNAs or average-sized mRNAs. During initial experiments with DC2.4 cells, we experienced problems with the transfection of replicon RNA. To search for an alternative to the earlier established electroporation, different methods, including novel techniques, were tested in order to achieve high transfection efficiencies and, at the same time, low cytotoxicity.
First, various different transfection reagents, in part specifically designed to transfect RNA, were compared with electroporation (Table 4.2). Both lipid-based transfection reagents (TransMessanger, fuGENE HD) failed to transfect replicon RNA into DC2.4 cells and the transfection with polymer-based reagents (Man-PEI, TransIT) was of very low efficiency. Even though electroporation was moderately cytotoxic, it was by far more efficient than the other methods and was thus the method of choice to transfect replicons in DC2.4 cells.

Table 4.2: List of reagents and methods tested for the transfection of replicon RNA in DC2.4 cells

The mono-cistronic replicon DI9c was used for the experiments and transfection efficiency was determined 24 h post transfection by immunohistochemistry staining for NS3. Cytotoxicity was determined by comparing the confluency of the cells to the respective untreated control. Transfection experiments using the listed reagents were performed according to protocols supplied by the manufacturers. Elelectroporation conditions were as follows: 300 V, 750 µF, 400 Ω, in a 4-mm-cuvette with 5 µg RNA in 200 µl PBS. -: no ; ++: low, +++: moderate cytotoxicity.

SYSTEM	CYTOTOXICITY	TRANSFECTION EFFICIENCY IN %
Man-PEI	++	< 1
TransMessanger	-	-
fuGENE HD	+++	-
TransIT	-	ca. 1
electroporation	+++	10-20

Interestingly, the efficiency of electroporation could be massively improved by the isolation of single DC clones. For this purpose, DC2.4 cells were sub-cloned by limiting dilution (see 3.2.2 Subcloning of DC2.4 cells by limiting dilution). That is, a high dilution of DC2.4 cells was prepared and plated in a 96-well plate to isolate individual cells. Cell clones with increased transfection efficiency were identified and subjected to an additional sub-cloning step. The procedure of cell clone isolation was repeated for a total of three times (Table 4.3), eventually increasing the fraction of replicon-transfectable cells to 34% in the population of clone 15/1/2. This clone was selected

for vaccination experiments and retained an average transfection efficiency of about 30%. Importantly, these clones were confirmed by FACS analysis to exhibit the same expression levels of the DC surface molecules CD86, CD80, CD40 (data not shown).

CELLS	TRANSFECTION EFFICIENCY IN %
Clone 3	14
Clone 15	23
Clone 15/1	28
Clone 15/2	26
Clone 15/1/2	34

Table 4.3: Transfection efficiency of DC2.4 cells and various sub-clones

Cells we.re transfected with 5 µg of Repl-IL12 by electroporation (see 3.2.2 Transfection of RNA replicons by electroporation); individual sub-cloning steps are separated by a slash.

4.1.3 Evaluation of replicons

Prior to using the RNAs for the vaccination of mice, it was important to test the bicistronic replicons encoding for the Her2 fragments or IL-12 in terms of replication capacity, the induction of apoptosis (cytopathic effect) and antigen expression in transfected cells.

Replication

The replication capacity of the respective RNA molecules was confirmed by immunofluorescence staining of the BVDV gene product NS3 in transfected cells. To this end, the DNA templates for RNA transcription were linearized by *Sma*I or *Srf*I, or by *Cla*I in the NS5A coding region. While in the former cases, transcription yielded the full-length viral RNA containing the correct 3'-terminus, transcription of the latter template yielded transcripts without the viral RNA's 3'end (Figure 4.7, A). Since RNAs which lack the 3'-region are replication-deficient, they served as a control. In other words, replication associated synthesis of NS3 was measured versus NS3 synthesis mediated solely by translation of the transfected RNA molecules.

The bovine MDBK cells were transfected with both transcripts, respectively, and stained for NS3 24 h post transfection. As expected, in the absence of RNA replication of the transfected RNA molecules, the NS3 concentration was below the detection limit of the assay (Figure 4.7, B). Conversely, cells transfected with the full-length replicons were positively stained for NS3, which confirmed that the respective RNA constructs had replication activity. A representative image is shown for Repl-rECD in Figure 4.7, C and D.

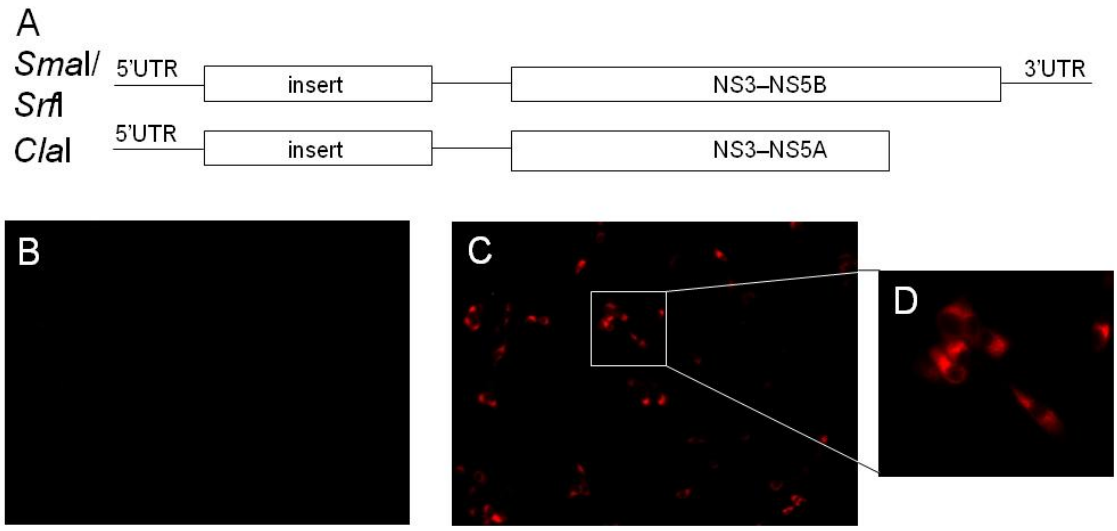

Figure 4.7: Replication of replicon RNA in transfected MDBK cells

Replicon template DNA was linearized with *Srf*I to obtain the full-length replicons or prematurely with *Cla*I generating truncated replicon RNA that was used as a negative control. Both RNA molecules were transfected into MDBK cells, respectively, and replication was determined by NS3-expression at 24 h post transfection. (A) Schematic view of full-length and truncated RNA. Image of immunofluoresence staining for NS3 of cells transfected with (B) truncated or (C) full-length Repl-rECD. (D) Detail of cells in the framed image section in C shows cytoplasmic expression of NS3.

To obtain an estimate on the relative replication efficiency of each of the individual replicon constructs, relative amounts of the BVDV gene product NS5A were quantified in replicon-transfected cells. To this end, cell lysates of replicon-transfected MDBK cells were collected at 24 h post transfection and analyzed by western blot. The signal intensity of NS5A staining was normalized to the signal of the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to account for differences in the amount of protein loaded on the gel.

As shown in Figure 4.8, differences in the amounts of NS5A in cells transfected with the various replicons were clearly detectable. Considering that for each construct the same amount of RNA was used for transfection and assuming that the BVDV NS-protein coding ORF should be expressed at the same level, the graph mirrors the relative replication efficiencies.

While only minor differences were observed between the *h*Her2-replicons, the replication efficiencies of RNA constructs encoding for the *r*Her2 fragments differed significantly. In particular, the Repl-rICD replicated at a notably lower level than Repl-rECD. The Repl-rMF and Repl-IL12 replicated at a slightly lower efficiency than Repl-rECD.

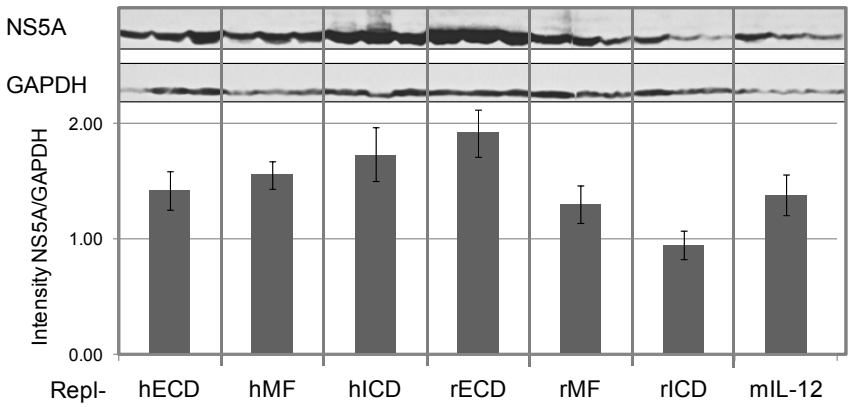

Figure 4.8: Replicons with different inserts vary in replication efficiency

Lysates from replicon-transfected MDBK cells were analyzed by western blot staining for BVDV-NS5A and GAPDH (upper part; for methods see 3.2.3 Western blot to compare relative replication efficiency). Signal intensity of NS5A staining was quantified and normalized against the GAPDH signal (bar graph; data were obtained from three transfections; error bar represents standard deviation).

Cytopathic effect

Since the time-delayed cytopathic effect was earlier shown to be crucial for antigen cross-presentation *in vivo* (Racanelli et al., 2004; see also Introduction section), all replicons were tested for the induction of cell death in transfected cells by flow cytometry. To further characterize the time course of the cytopathic effect, DC2.4 cells were transfected with Repl-rMF and stained using a dead cell marker and NS3 antibody at 15 h, 35 h and 51 h post transfection (Figure 4.9).

At 15 h post replicon transfection matching the time point, when the cells should be injected into mice during the intended vaccination experiments, low expression levels of NS3 were detectable. About 10% of the total population consisted of dead or dying cells, which was explained by the electroporation procedure. At 35 h post transfection, the expression level of NS3 was considerably higher and about 40% of the transfected cells (NS3-positive) were dying or dead, demonstrating replicon-induced cell death. At 51 h post transfection, only a few vital cells expressing NS3 remained detectable. These results show that the majority of transfected cells died in the observed time course.

Importantly, all seven replicon constructs were further confirmed to cause a cytopathic effect as determined by dead cell staining with propidium iodide at 24 h and 48 h post transfection, respectively (data not shown).

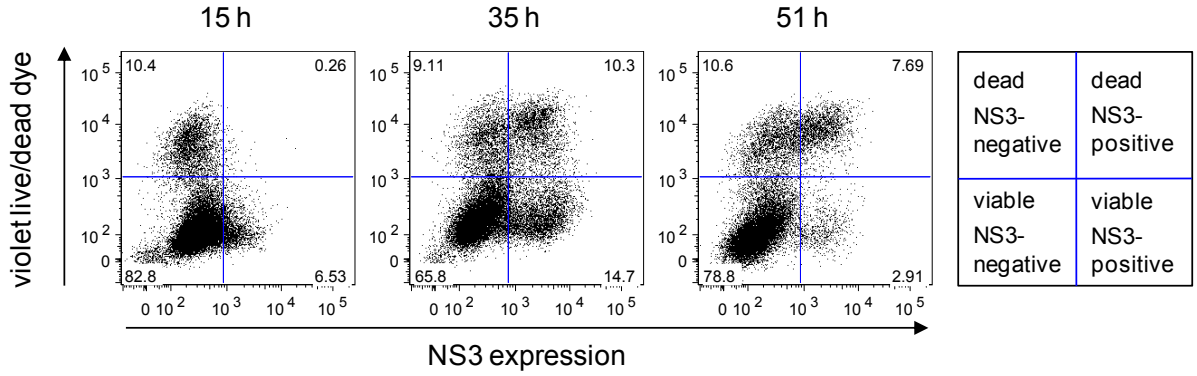

Figure 4.9: Replicons mediate time-delayed cell death of transfected DC2.4 cells

Dotplots of the FACS analysis of cells transfected with Repl-rMF are shown at 15 h, 35 h and 51 h post transfection. Cells were stained with a fluorescent dead cell detection reagent (violet live/dead dye, y-axis) and a combination of NS3 antibody and a fluorescently labeled secondary antibody (x-axis) to identify transfected cells. Fluorescence intensity of the two stainings is plotted on the respective axes. The quadrant gate (blue lines) is based on the four separate populations of the 35 h sample.
At 15 h post electroporation, transfected cells started to express NS3 (lower right quadrant). Expression level of NS3 was increased at 35 h and about 40 % of the transfected cells were co-stained with the dead cell marker (upper right quadrant). At 51 h post transfection, only few transfected cells were viable (lower right quadrant).

Antigen Expression

Next, it was important to confirm the expression of the protein encoded by the respective replicon inserts. Translation of the encoded antigens was analyzed by western blot, *in vitro* translation and ELISA.

Western Blot

One approach to detect expression of the encoded antigens was the western blot analysis of cell lysate from replicon-transfected cells. Thus, expression of *m*IL-12 was confirmed by direct western blotting using an antibody directed against the FLAG epitope (Figure 4.10, A). With lysates of Repl-IL12 transfected cells, a specific protein band was detected that migrated at the theoretical molecular weight of *m*IL-12 (see Table 4.4). Conversely, none of the Her2 antigens were detectable by western blotting of the lysates of Repl-hMF-, Repl-hICD-, Repl-rMF- and Repl-rICD-transfected cells. For that reason, the FLAG-tagged proteins were first concentrated by immunoprecipitation using the aforementioned anti-Flag antibody. Testing this immunoprecipitate by western blot, specific protein bands were detected that exhibited an SDS migration pattern corresponding to the theoretical molecular weight of *h*ECD and *r*ECD, respectively (see Table 4.4) (Figure 4.10, B). Thus, expression of *m*IL-12, *h*ECD and *r*ECD in replicon-transfected cells was demonstrated. However, *h*MF, *h*ICD, *r*MF and *r*ICD were not detectable with either of the methods.

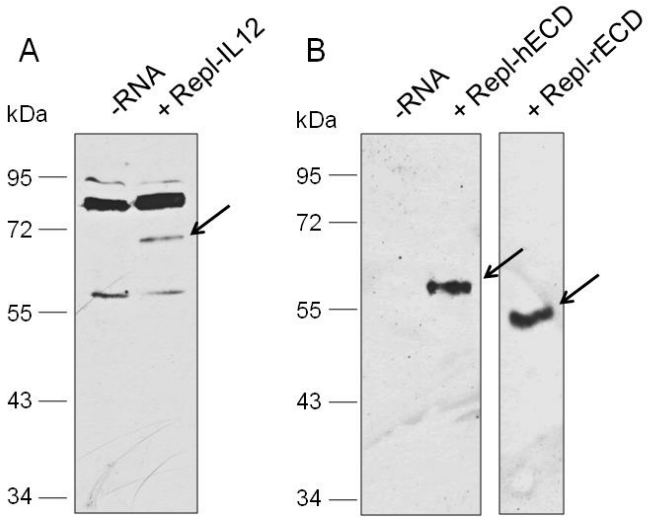

Figure 4.10: *m*IL-12, *h*ECD and *r*ECD are expressed from the corresponding replicons in cells

Western blot of protein that was separated by SDS-PAGE (10%). (A) Cell lysates of mock-transfected (-RNA) and Repl-IL12-transfected MDBK cells. Note that Repl-IL12-transfected cells display a specific protein band that has the theoretical molecular weight of *m*IL-12 (arrow). (B) FLAG-tagged proteins were concentrated from cell lysates of Repl-hECD- and Repl-rECD-transfected MDBK cells by immunoprecipitation with anti-Flag antibody and subjected to a western-blot using the same antibody. Notably, the molecular weight of the detected protein bands corresponded to the theoretical molecular weight of the respective Her2 antigenic protein fragments (arrows) whereas no band was detected using mock-transfected cells.

In vitro translation

Because antigen expression could not be demonstrated for all constructs by western blot or immunoprecipitates of the cell lysates, *in vitro* translation of the replicons was performed. That is, the *in vitro* transcribed replicon RNAs were translated in Huh7 cell extracts supplemented with S-35-labeled methionine and cysteine. The radioactively labeled proteins were subsequently analyzed by SDS PAGE. This method has an increased detection sensitivity compared to western blot. Since the assay was not specific for the heterologous proteins, protein expression by the bi-cistronic replicon constructs was compared to that of the mono-cistronic BVDV replicon DI9c (see Figure 2.4). In this way, it was possible to discriminate between the diverse BVDV proteins and the heterologous translation products.

As shown in Figure 4.11, translation of the BVDV proteins NS3 and N^{PRO} was, as expected, detectable in each case, i.e. with the mono-cistronic and the bi-cistronic replicons. However, in comparison to the mono-cistronic replicon, two additional protein bands were detected in the translation experiments with Repl-hMF and Repl-rMF. The migration pattern corresponded to the molecular weight of (i) the MF fragment and (ii) the sum of MF fragment and the N^{PRO} protein (Table 4.4, Table 4.5, Figure 4.11).

Thus, while expression of the *h*MF and *r*MF peptides could be detected for the first time, the proteolytic reaction was incomplete during *in vitro* translation, which resulted in the uncleaved fusion protein (N^{PRO}-MF) and the individual proteins (N^{PRO} and MF).
The same migration pattern, i.e. two protein bands corresponding to the translation product of the replicon insert and a precursor consisting of the heterologous protein and N^{PRO}, were observed for the translation assays with each of the ECD replicons but not with any of the two ICD replicons.

Table 4.4: Calculated molecular weights of heterologous proteins and BVDV proteins in kDa

heterologous protein	*h*ECD	*h*MF	*h*ICD	*r*ECD	*r*MF	*r*ICD	*m*IL-12	NS3	N^{PRO}
size (kDa)	58	71	65	50	68	68	61	75	19

Table 4.5: Calculated molecular weights of N^{PRO}-insert fusion proteins in kDa

fusion protein	*h*ECD+ N^{PRO}	*h*MF+ N^{PRO}	*r*ECD+ N^{PRO}	*r*MF+ N^{PRO}
size (kDa)	77	80	69	87

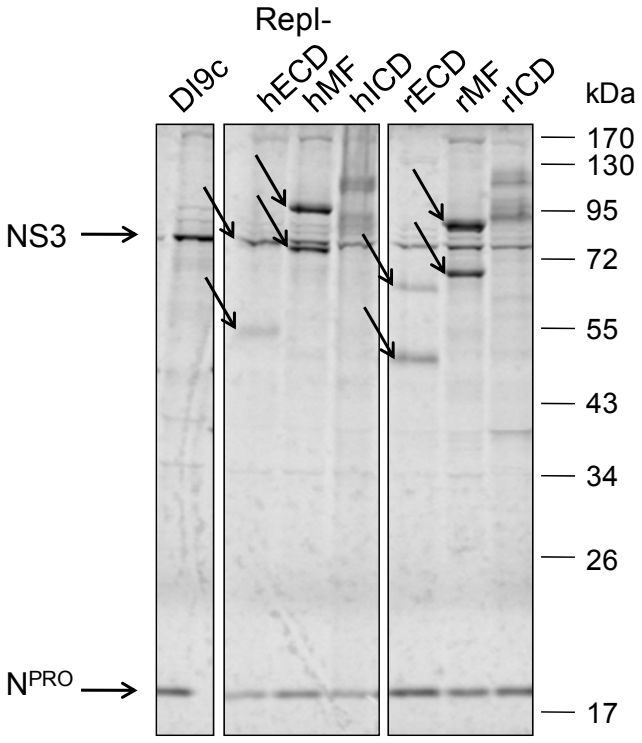

Figure 4.11: ECD and MF Her2 fragments are expressed from the corresponding replicons

The phosphorimager gel image shows proteins produced by *in vitro* translation of various replicons. While the BVDV proteins NS3 and N^{PRO} (arrows) were translated from the bi-cistronic constructs as well as from the monocistronic replicon (DI9c), additional bands indicate expression of the heterologous insert in the bi-cistronic constructs. As explained in Table 5-2, the molecular weights of these additional protein bands correspond to that of the additionally encoded Her2 protein fragment and a precursor of the Her2 protein fragment and N^{PRO}, respectively. Note that heterologous protein expression was detectable for the ECD and MF replicons but not the ICD replicons.

In conclusion, these data showed that *h*ECD, *h*MF, *r*ECD and *r*MF were definitely expressed from the corresponding replicon-constructs. However, it was not possible to

show expression of *h*ICD and *r*ICD, neither by western blot analysis, nor by *in vitro* translation.

Detection of secreted IL-12 by ELISA

To verify the functionality of Repl-IL12, it was important to show that the biologically active form of the cytokine is produced and released by transfected cells in order to mediate its effector function during vaccination. To this end, the supernatants of cells, that had been transfected with Repl-IL12, were collected at 14 h, 24 h and 48 h post transfection and the concentration of IL-12 was determined by an ELISA that was specific for the bioactive form of the cytokine (Figure 4.12; see Materials and Methods 3.2.3).

Interestingly, IL-12 was already detectable in the supernatant at 14 h post transfection. That is, at a time point at which all transfected cells were viable (see Figure 4.9) IL-12 was actively secreted in the medium. The IL-12 concentration increased about 6-fold between 14 h and 24 h post transfection, which coincides with the production of high levels of replicon-encoded proteins. At 48 h post transfection, the concentration of IL-12 further increased, probably due to an accumulation of the protein in the supernatant and related to the fact that most cells had already died at this time point, which might have led to the release of intracellular IL-12. In contrast, IL-12 expression was undetectable in transfection experiments with Repl-rMF, which were performed as controls.

In sum, these data demonstrate that IL-12 was produced in its bioactive form and released into the medium of Repl-IL12-transfected cells.

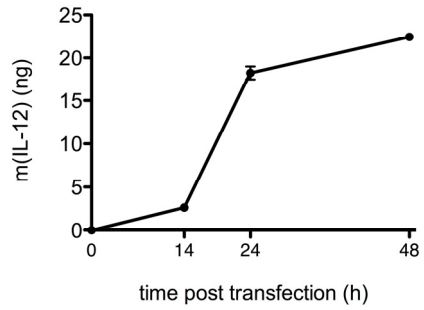

Figure 4.12: IL-12 is produced and released from cells transfected with Repl-IL12

Cell culture supernatants were collected at 14 h, 24 h and 48 h post transfection and analyzed by ELISA (see text). Data was obtained from three independent experiments (Mean ± standard deviation).

4.1.4 Summary

Summarizing the first part of this thesis studies, it has been possible to generate and characterize cDNA templates of bi-cistronic BVDV RNA replicons that encode the ECD, MF and ICD fragments of *h*Her2 and *r*Her2 as well as IL-12, respectively. The DC line DC2.4 was tested for different transfection procedures, and novel DC-clones were established that allowed transfection efficiencies of up to ca. 30%. All cDNA-derived replicon RNAs were shown to replicate and to mediate a time-delayed cytopathic ef-

fect in different types of transfected cells. Expression of the heterologous proteins was demonstrated for the Her2-ECD and Her2-MF fragments as well as for IL-12 but not for Her2-ICD. It was further demonstrated that functional IL-12 was released into the culture supernatant of IL-12 replicon transfected cells.
The observed differences in the detection of antigen expression levels were explained by the nature of the inserts. That is, the IL-12 replicon encodes the entire gene resulting in the translation of the complete protein that contains all information for proper folding. In contrast, the Her2 replicons encode only fragments of the full-length protein, therefore correct folding is not guaranteed. As a result, the nascent polypeptides might be vulnerable to enzymatic digestion. In other words, the individual Her2 fragments may have different stabilities, which might explain why both ECD fragments but none of the other Her2 fragments were detected after concentration.
Regarding the effect of antigen instability on the vaccination efficiency, it was considered unlikely having an impact on antigen presentation as long as the antigens were expressed. In contrast, rapid degradation of the antigens might even improve presentation of antigen peptides, because MHC class I epitopes are naturally processed from incomplete or degraded translation products. Thus unstable antigens may even have increased antigenicity (Yedwell & Nicchitta, 2006). In fact, strategies have been developed to deliberately destabilize antigens within DCs in order to improve vaccination efficiency (Leifert et al., 2004; Hosoi et al., 2008).
In the second part of the thesis, DC2.4 cells transfected with the Her2-ECD, Her2-MF and IL-12 replicons were used to immunize mice (procedure described in Materials and Methods 3.2.4). To investigate potential anti-tumor effects of the vaccination, mice were challenged with tumor cells that over-express the *r*Her2 antigen. Accordingly, replicons that encoded fragments of *r*Her2 were chosen for vaccination. However, since Repl-rICD could not be verified for antigen expression, this construct was excluded from the vaccination experiments.

4.2 Anti-tumor vaccination

The anti-tumor effect of the vaccination with replicon-transfected DCs was analyzed in a tumor challenge model. Along this line, FVB/N mice were subcutaneously vaccinated with *r*Her2-expressing tumor cells (NT-2 cells, kindly provided by E. Jaffee, Reilly et al., 2000), which lead to tumor growth in non-vaccinated mice (Figure 4.14, black line). The *in vivo* effect of the vaccination was tested in two different models to evaluate the potential of the vaccination: i.e., (i) whether the procedure prevented tumor growth (preventive vaccination, Figure 4.13) and (ii) whether it affected already established tumors (therapeutic vaccination, Figure 4.16).
Of note, FVB/N mice were used in this study to evaluate particularly the role of cross-priming in the vaccination. Because DC2.4 cells (H-2^b) have a different MHC haplotype than FVB/N mice (H-2^q), T cells cannot recognize MHC-peptide complexes on

DC2.4 cells. Consequently, Her2-specific T cells in FVB/N mice are induced solely by cross-priming.

4.2.1 Preventive vaccination

For the preventive vaccination, mice were vaccinated twice with DC2.4 cells transfected with the single replicons, Repl-rECD, Repl-rMF or Repl-IL12 (referred to as ECD, MF or IL-12 group), or a combination of Her2-replicons and Repl-IL12 (ECD/IL-12, MF/IL-12 groups). Subsequently, the animals were challenged with tumor cells. Mock-transfected DC2.4 cells served as a negative control.

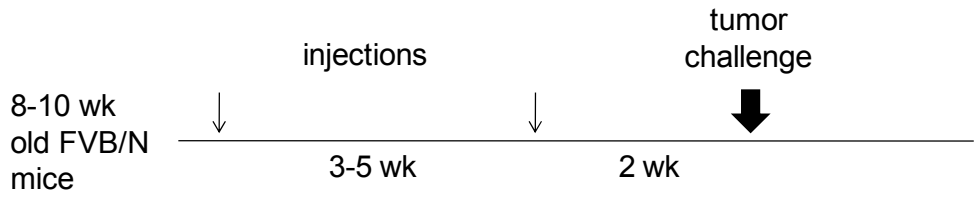

Figure 4.13: Experimental design of the preventive vaccination

Mice were vaccinated twice by injection of replicon-transfected DC2.4 cells. The chosen time intervals of vaccination were 3–5 weeks (wk). Challenge with *r*Her2-expressing tumor cells was performed at wk 2 after the second vaccination. Thin arrows: injection of replicon- or mock-transfected DCs. Bold arrow: tumor challenge (injection of tumor cells).

Average tumor size

The effect of vaccination on tumor growth was analyzed by determining the average tumor size over the time post tumor challenge (Figure 4.14, A).

As shown in Figure 4.14, mice vaccinated with DCs that were transfected with Her2-encoding replicons developed significantly smaller tumors than the mock-vaccinated mice (ECD: p=0.0086, MF: p=0.0127, ECD+IL-12: p=0.011; MF+IL-12: p=0.001) (Figure 4.14, B). In contrast, vaccination with a replicon such as Repl-IL12, that did not encode any Her2 antigens, did not significantly decrease tumor size. Note that no significant differences were observed between vaccinations with replicons encoding the two different fragments of Her2, ECD and MF.

Protection against tumor growth

In addition to the analysis of the mean tumor size, the number of mice that were protected against tumor growth by the end of the experiment was determined (Figure 4.15). Tumors did either not develop at all or an initially growing tumor was eventually eradicated in these mice.

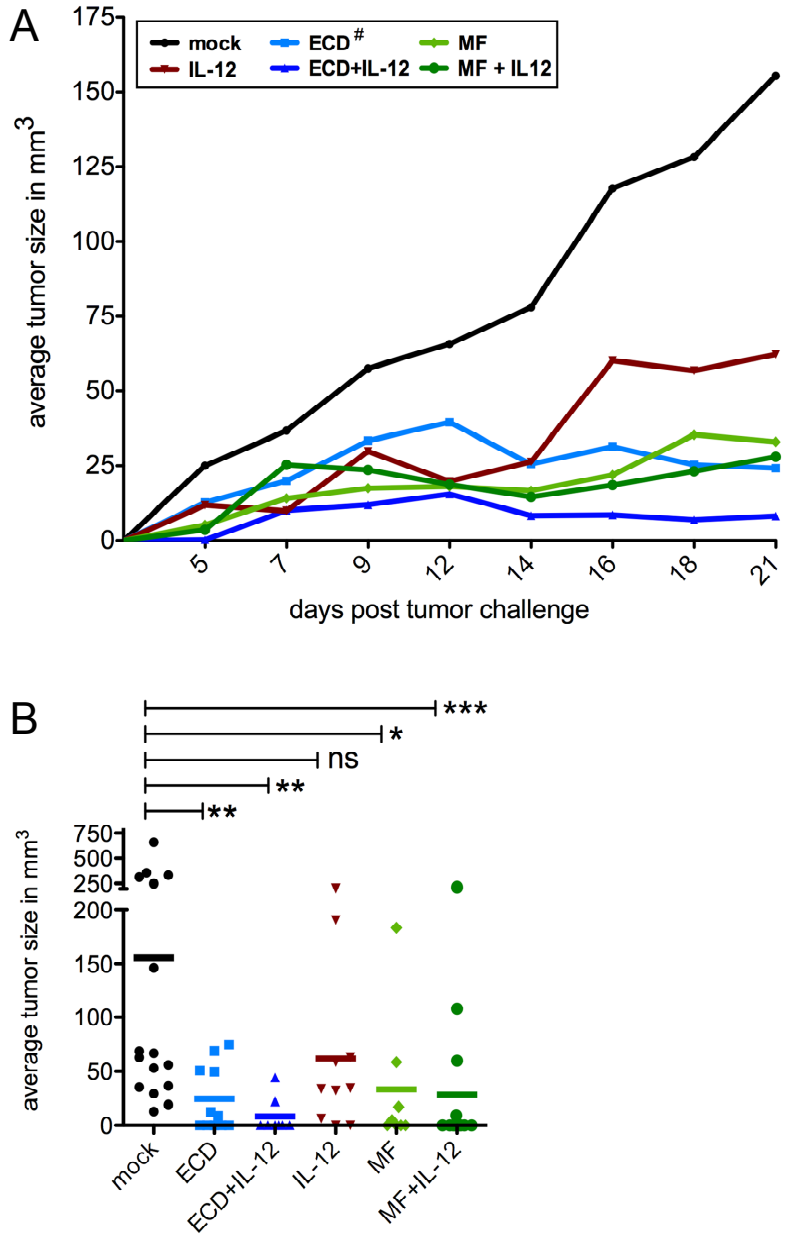

Figure 4.14: Anti-tumor effect observed after preventive vaccination

Mice were vaccinated first with replicon- or mock-transfected DC2.4 cells and then challenged with Her2-expressing tumor cells (day 0). (A) The average tumor size of each group was determined over three weeks post tumor challenge. (B) The mean tumor size on day 21 is shown in a vertical scatter plot and compared between the experimental mouse groups and the mock-vaccinated group. Data is shown for three independent experiments. Number of mice per group: mock: n=16, ECD: n=11, MF: n=8, IL-12: n=10, ECD+IL-12: n=8, MF+IL-12: n=14; ns – not significant; *– significant (P: 0.01–0.05); **– very significant (P: 0.001–0.01); ***– extremely significant (P<0.001); #One of 12 mice in the ECD group was excluded from analysis of the average tumor size because the tumor volume was about 6.5 times higher than the average of the mock group.

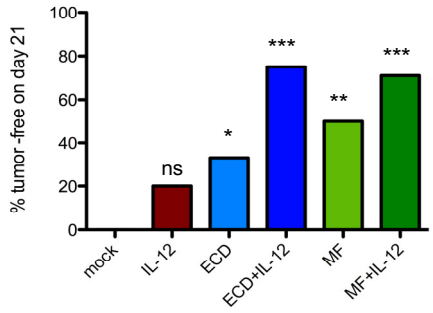

Figure 4.15: Protection against tumor growth by preventive vaccination

Mice were vaccinated with replicon- or mock-transfected DC2.4 cells and then challenged with Her2-expressing tumor cells. The number of tumor-bearing mice was determined on day 21 post tumor challenge and the fraction of tumor-free mice was calculated. Number of mice per group: mock: n=16, ECD: n=12, MF: n=8, IL-12: n=10, ECD+IL-12: n=8, MF+IL-12: n=14. ns – not significant; *– significant (P: 0.01–0.05); **– very significant (P: 0.001–0.01); ***– extremely significant (P<0.001)

Consistent with the analysis of the average tumor size, vaccination experiments with Her2-replicon-transfected DCs mediated a significant anti-tumor response (ECD: p=0.0242, MF: p=0.0066, ECD+IL-12: p=0.0002; MF+IL-12: p<0.0001). That is, 33% of mice in the ECD group and 50% of mice in the MF group were completely tumor-free by the end of the experiment. In contrast, all 16 mice in the mock group developed tumors. Co-administration of DCs transfected with Repl-IL12 and either of the Her-2 replicons showed a trend towards better protection against tumor growth compared to the respective vaccination groups (ECD+IL-12: 75% vs. ECD: 33%, MF+IL-12: 71% vs. MF: 50%). In contrast, administration of DCs transfected with Repl-IL12 alone protected only 20% of the mice, which was not significantly different from the data obtained with the mock group. Again, no differences were observed between vaccinations with the ECD or MF replicons.

4.2.2 Therapeutic vaccination

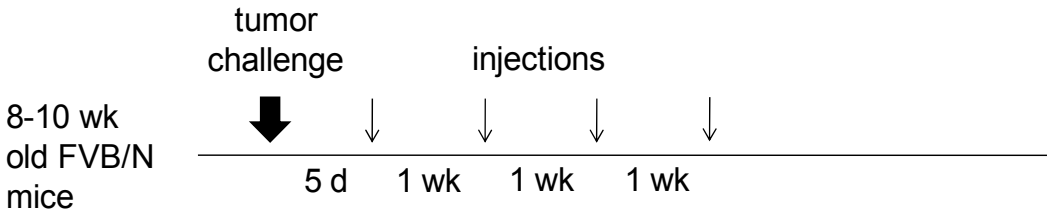

Figure 4.16: Experimental design of the therapeutic vaccination

Mice were challenged with Her2-expressing tumor cells prior to vaccination. The first injection of DC2.4 cells was performed when tumors were visible, followed by additional three vaccinations at a time interval of 1 wk.Thin arrows: injection of replicon- or mock-transfected DCs. Bold arrow: tumor challenge (injection of tumor cells).

To analyze the efficacy of vaccination against already established tumors, FVB/N mice were first challenged with the tumor cells and then vaccinated. Vaccination with replicon-transfected DCs was started when all mice had visible tumors, which was five days post tumor challenge. The vaccination was repeated every week for a total of four vaccinations and tumor growth was monitored during this time (Figure 4.16).
Since co-administration of Repl-rMF- and Repl-IL12-transfected DCs mediated a significant anti-tumor effect in the preventive vaccination protocol, this procedure was tested for therapeutic vaccination. The effect of this regimen was compared to vaccination with Repl-IL12- and mock-transfected DCs, respectively.
Figure 4.17 shows that in all three groups the tumor size constantly increased over time. Even after four vaccinations, a therapeutic effect of the vaccine was not detectable. Furthermore, injection of replicon-transfected DCs did not induce a detectable decrease in the tumor growth rate. Hence, vaccination with replicon-transfected DCs had no effect on established tumors.

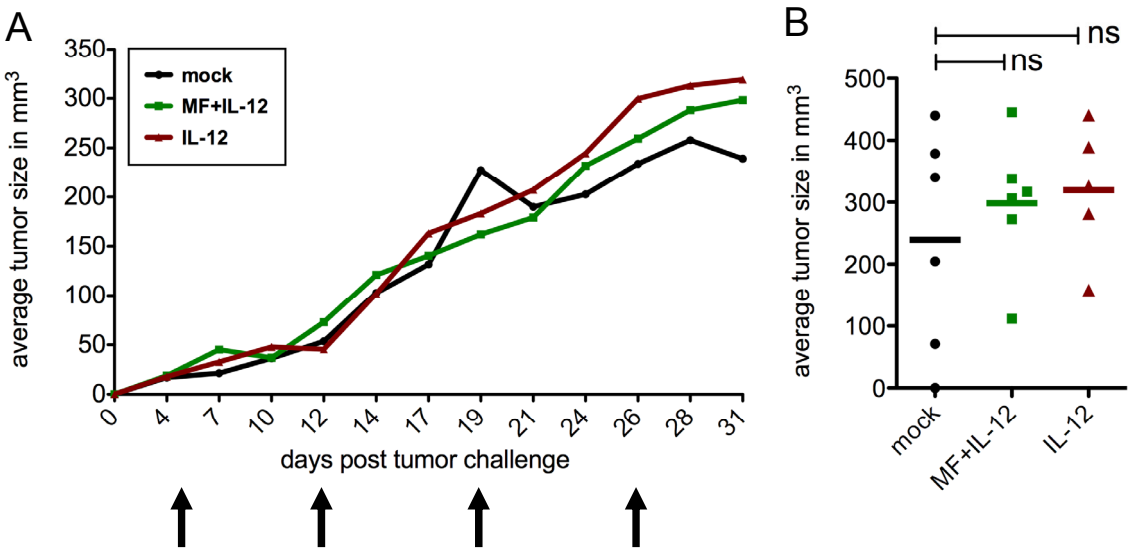

Figure 4.17: Therapeutic vaccination with replicon-transfected DCs

Mice were challenged with tumor cells on day 0. Vaccination was initiated five days post tumor challenge and repeated 3 times with a 3-week time interval between the injections. (A) Average tumor size of each group was determined over 31 days. Black arrows indicate time of vaccinations. (B) Tumor sizes on day 31 after vaccination in the respective vaccination groups is presented in a vertical scatter plot. Number of mice: mock: n=6, MF+IL-12: n=6, IL-12: n=5. ns: not significant

A similar trend was observed for therapeutic vaccination of mice with Repl-rECD with or without Repl-IL12 (Figure 4.18). However, it should be noted that in this experiment, the sample number was low, and vaccination was started as late as 9 days post tumor challenge.

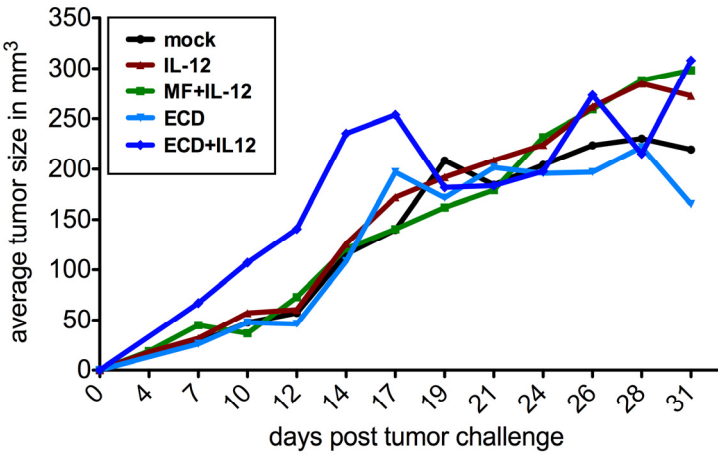

Figure 4.18: None of the replicon constructs mediates an anti-tumor response in therapeutic vaccination experiments

Mice were challenged with tumor cells on day 0 and vaccination started 5 days (mock, IL-12, MF+IL-12) or 9 days (mock, IL-12, ECD, ECD+IL-12) later and was repeated every week for a total of 4 times. Tumor size was measured over 31 days post tumor challenge. The mean tumor size for each group is displayed. Number of mice: mock: n=8, IL-12: n=9, MF+IL-12: n=6, ECD: n=4, ECD+IL-12: n=4. The data of two separate experiments is combined (including the experiment shown in Fig. 5-17).

4.2.3 Summary

To summarize, vaccination with Her2 replicon-transfected DCs was demonstrated to mediate a significant anti-tumor effect in a preventive vaccination approach. Along this line, Repl-rECD and Repl-rMF turned out to be equally efficient, and co-administration of Repl-IL12 did not significantly increase the efficacy of the vaccination, though a trend to more mice being completely protected against tumor growth was observed. In contrast, vaccination did not affect tumor growth when administered as a therapeutic vaccine after tumors had already established (see Discussion 5.1.5 Preventive vs. therapeutic vaccination).

The similar vaccination efficacy of both Her2 replicons might be due to one or more epitopes that are encoded by both fragments. Specifically, the immunodominant epitope 420–429 (Ercolini et al., 2003) is located in the overlapping sequence of ECD and MF and might be important for the anti-tumor immune response that is induced by vaccination (see Figure 4.2).

4.3 Immune response

The preventive vaccination approach demonstrated a significant anti-tumor response after vaccination with DCs transfected with Her2 replicons, which was not observed after vaccination with Repl-IL12. This observation led to the conclusion that the anti-tumor effect was mediated by specific activation of the immune system against the

tumor antigen. It was next important to determine the components of the immune system that account for the anti-tumor effect. In particular, the cellular (CD4+ and CD8+ T cells) and humoral (antibodies) immune response should be analyzed.

4.3.1 Role of CD4+ and CD8+ T cells in mediating the anti-tumor response

To study the role of T lymphocytes for the *in vivo* vaccination effect, tumor growth was monitored in the absence of CD4+ or CD8+ T cells. Mice were vaccinated with the combination of Repl-rMF- and Repl-IL12-transfected DCs following the protocol of preventive vaccination. However, T cell subsets were depleted in the vaccinated animals prior to and during tumor challenge by injecting depletion antibodies (Figure 4.19).

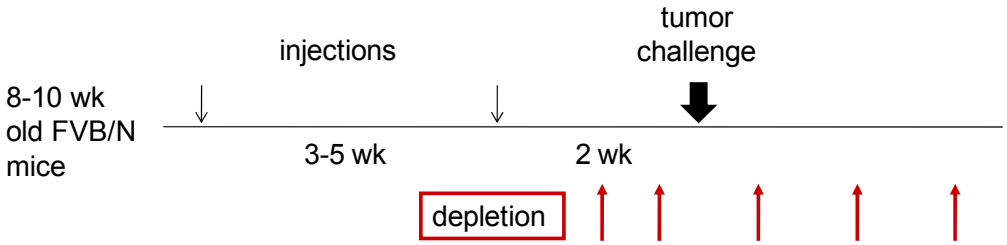

Figure 4.19: Experimental design of the depletion experiment

Mice were vaccinated according to the protocol applied during preventive vaccination (see Figure 4.13). CD4 or CD8 depleting antibodies or PBS were injected i.p. before and during tumor challenge to deplete the respective T cell subset. Thin, black arrows: injection of DCs transfected with Repl-rMF- and Repl-IL12. Bold, black arrow: tumor challenge (injection of tumor cells). Red arrows: injection of PBS or depletion antibodies.

The efficiency of T cell subset depletion was determined on the day before tumor challenge (Figure 4.20) and at the end of the experiment. After two injetions of the depleting antibodies, 99.91% of CD4+ T cells and 99.01% of CD8+ T cells were depleted in the mice treated with the respective antibody. At the end of the experiment, an average of 99.99% of CD4+ T cells and 99.95% of CD8+ T cells compared to untreated mice were depleted in splenocytes of mice treated with CD4 antibodies and CD8 antibodies, respectively.

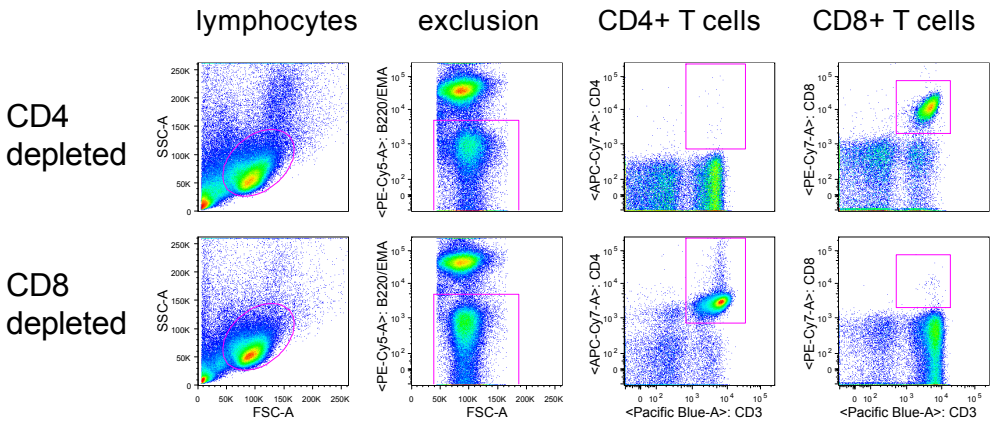

Figure 4.20: T cell subsets are successfully depleted

The dotplots show the FACS analysis of splenocytes from depleted mice on day -1 of tumor challenge. Splenocytes were isolated from a CD4- and a CD8-depleted mouse, respectively, and the red blood cells were lyzed. Subsequently, the cells were stained for various surface markers and analyzed by flow cytometry. Total cells were gated for lymphocytes, followed by the exclusion of B cells (B220+) and dead cells (EMA+). T cell subsets were identified by the expression of CD3 and CD8 or CD4, respectively. PE-Cy-5, PE-Cy7, APC-Cy7, Pacific Blue: fluorescence molecules conjugated to the respective antibody. EMA: ethidium monoazide. Pink circles and squares identify the cell population of interest.

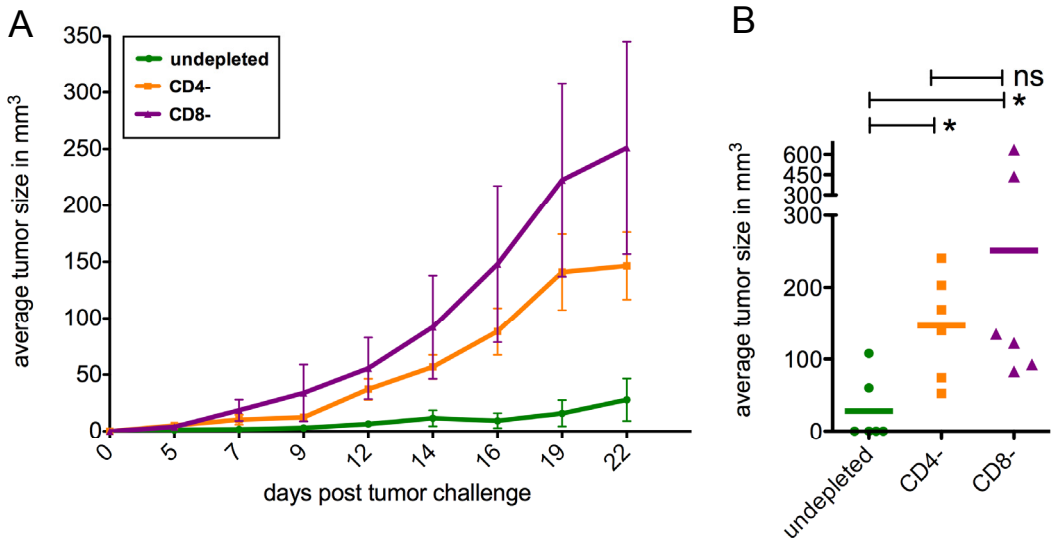

Figure 4.21: Both, CD4+ and CD8+ T cells contribute to the vaccine-induced anti-tumor effect

Mice were vaccinated with Repl-rMF- and Repl-IL12-transfected DCs and left untreated (undepleted) or treated (injected) with CD4 antibodies (CD4-) and CD8 antibodies (CD8-), respectively, before and during tumor challenge. (A) Mean tumor size (± standard error of the mean) for each group is displayed over 22 days. (B) Mean tumor size on day 22 is shown in a vertical scatter plot and compared between the groups. Number of mice: 6 in each group. ns – not significant; *– significant (P: 0.01–0.05);

Consistent with the results shown in Figure 4.14, mice vaccinated with Repl-rMF- and Repl-IL12-transfected DCs were mainly protected against tumor growth. That is, undepleted mice developed only small tumors upon tumor challenge (Figure 4.21, A, green line) and four out of the six mice were protected against tumor growth (Figure 4.21, B). In contrast, both, CD4- as well as CD8-depleted mice showed an increase in tumor size over time and all mice developed tumors (see also Figure 4.21, A and B). Moreover, the average tumor size on day 22 was significantly higher when compared to those of undepleted mice (CD4 depletion: p=0.0181, CD8 depletion: p=0.0115) demonstrating that both T cell subsets are important in the vaccine-induced immune response. Another interesting result worth mentioning is that CD8 depleted mice showed a trend towards larger tumors as compared to the CD4 depleted group. However, the observed difference was not significant.

4.3.2 CD8+ T cell response

Activation of cytotoxic T lymphocytes is important for cancer immunotherapy as endogenous tumor antigens are generally presented via MHC class I. In congruence with this idea, the above depletion experiments of CD8+ cells demonstrated that CD8+ T cells are crucial for the replicon vaccine-induced immune response against Her2 cancer cells (see 4.3.1). To next determine whether the vaccination with DCs transfected with cytopathic replicons cross-primed Her2-specific CD8+ T cells, CD8+ cells from vaccinated mice were analyzed in an IFN-γ ELISpot assay. Specifically, CD8+ cells were separated from splenocytes of mice from the preventive vaccination experiment and stimulated with Her2 peptides *in vitro*. The number of Her2-specific cells was determined by counting spots positively stained for IFN-γ (spot-forming units, SFU). A typical image of an ELISpot plate with CD8+ cells from Repl-rECD-vaccinated mice shows, that IFN-γ production was specifically induced by the stimulation with Her2 peptides (overlapping peptides, OLP)(Figure 4.22).

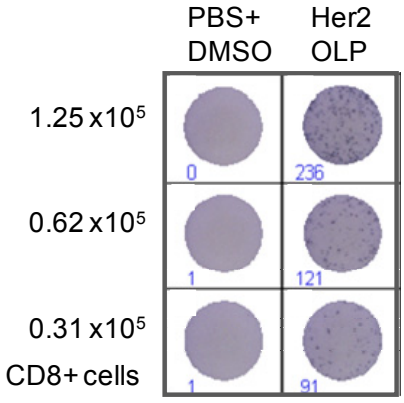

Figure 4.22: ELISpot assay measuring the Her2-specific CD8+ T cell response

CD8+ cells were isolated from splenocytes of a Repl-rECD-vaccinated mouse and stimulated with peptides of the Her2 antigen (OLP: overlapping peptides) or the corresponding diluent (PBS+DMSO). Wells were stained for IFN-γ to enumerate the number of activated cells (for details, see Materials and Methods 3.2.2).

As expected, mice that were not vaccinated with Her2-encoding replicons, namely the mock group and the IL-12 group, demonstrated an overall weak CD8+ T cell response (Figure 4.23, A). Accordingly, these two groups displayed the largest tumors by the end of the preventive vaccination (see Figure 4.14). Based on the data obtained for the mock and IL-12 vaccination groups, a strong CD8+ T cell response was defined as more than 100 SFU per $1.25x10^5$ CD8+ cells. Following this definition, only 1 out of 12 mice (8%) in the non-Her2 replicon-vaccinated groups clearly developed Her2-specific CD8+ T cells (mock, M1).

Conversely, as compared to the mock and IL-12 groups, significantly more mice in the groups vaccinated with Her2-encoding replicons showed a strong CD8+ T cell response (13 out of 23, 57%; p=0.0097) (Figure 4.23, B). In particular, vaccinations with a combination of Repl-rMF and Repl-IL12 turned out most efficient in inducing Her2-specific CD8+ T cells, because all 6 analyzed mice displayed a strong CD8+ T cell response (ECD: 3/8, 38%; ECD+IL-12: 2/4, 50%; MF: 2/5, 40%; MF+IL-12: 6/6, 100%).

Correlating the CD8+ T cell responses of vaccinated mice with their respective tumor sizes implies that a strong CD8+ T cell response protects against tumor growth (see, for example, mice from the ECD+IL-12 group in Figure 4.23, B). Indeed, the two parameters were inversely correlated (correlation tumor size and SFU for $0.62x10^5$ cells: r=-0.6017 with p=0.0001; correlation tumor size with SFU for $1.25x10^5$ cells: r=-0.4524 and p=0.0072; r: Spearman correlation coefficient).

This result strongly suggests that the vaccination with replicon-transfected DCs induced CD8+ T cells that are specific for the encoded antigen. Since DC2.4 cells and FVB/N mice have different MHC haplotypes, it is apparent that CD8+ T cells were induced by cross-priming. Based on the significant inverse correlation between CD8+ T cell response and tumor size, it can be concluded that the cross-priming of tumor antigen-specific CD8+ T cells is a major mechanism underlying the anti-tumor response induced by replicon-transfected DCs.

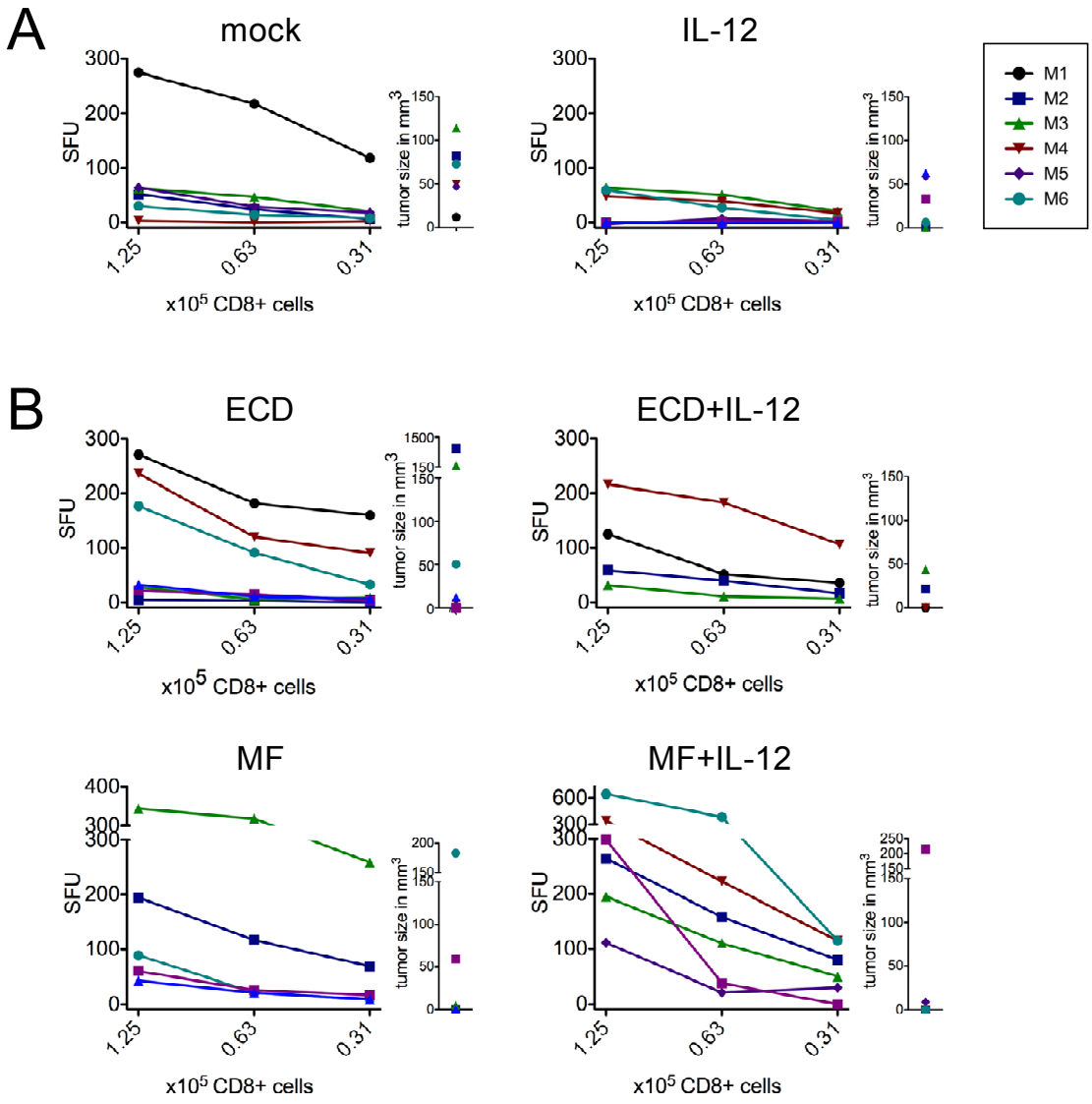

Figure 4.23: Vaccination with Her2-encoding replicons induces Her2-specific CD8+ T cells

CD8+ T cell responses were analyzed with an IFN-γ ELISpot assay. CD8+ splenocytes from mice of the preventive vaccination experiment were plated at 1.25, 0.63 and 0.31 x10^5 cells per well and stimulated with Her2 peptides (OLP). Her2-specific cells produced IFN-γ in response to the antigen and were enumerated by counting spots forming units (SFU). To compare the CD8+ T cell response with the tumor size in individual mice, the tumor size on day 21 post tumor challenge is shown for each group on the right of each ELISpot result. (A) Only 1 out of 12 mice that were vaccinated with non-Her2 replicon-transfected DCs (mock and IL-12) demonstrated a strong Her2-specific CD8+ T cell response. (B) 13 out of 23 mice that were vaccinated with Her2-replicons developed a strong Her2-specific CD8+ T cell response. Note that vaccination with a combination of Repl-rMF and Repl-IL12 was most efficient in inducing Her2-specific CD8+ T cells. For most mice, a strong T cell response coincided with protection against tumor growth or development of small tumors and vice versa. The number of SFU for mice M5-M8 of the ECD group and mice M5-M8 of the IL-12 group were multiplied by 2.2, and the number of SFU for mice M5-M7 of the MF group and mice M5-M7 of the MF+IL-12 group were multiplied by 4.3 to account for assay variability (see3.2.2, ELISpot assay).

4.3.3 Antibody response

The anti-tumor effect of the vaccination was reduced by the depletion of CD8+ cells, but also by the depletion of CD4+ cells (see 4.3.1). CD4+ T cells can differentiate in T_H1, T_H2 and T_H17 cells. T_H2 cells provide help for B cells to proliferate and to produce antibodies (Abbas et al., 2007). To test whether antibodies were involved in the observed anti-tumor effect, the humoral immune response against Her2 was analyzed. Plasma was obtained from vaccinated mice ten days after the second injection and was incubated undiluted with 3T3 cells expressing *r*Her2 (3T3/neu). Binding of antibodies to the cell surface containing the Her2 receptor was detected with a fluorophore-conjugated secondary anti-mouse IgG antibody. As a control for unspecific binding, plasma was incubated with Her2-negative 3T3 cells (3T3wt). Plasma from mice vaccinated with mock-transfected DCs served as a negative control and a monoclonal anti-*r*Her2 antibody served as a positive control.

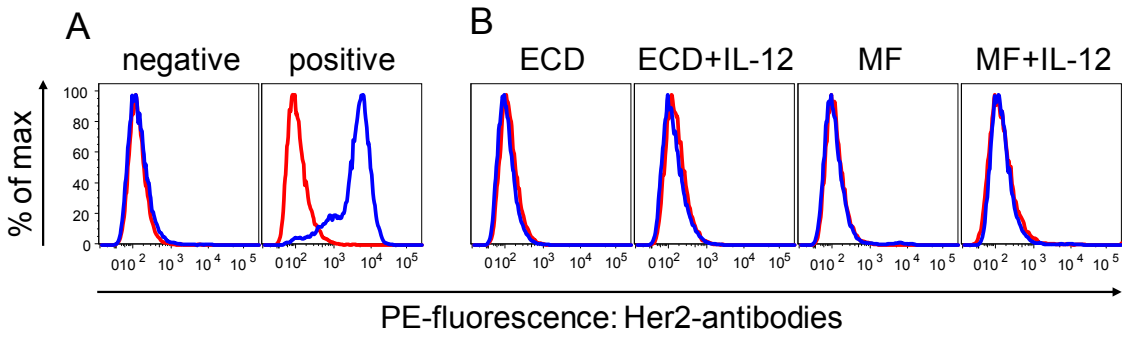

Figure 4.24: Vaccination does not induce Her2-specific antibodies

Plasma from mice was incubated with 3T3wt (red) and the Her2-expressing 3T3/neu (blue) cells and antibodies bound to the cells were stained with a PE-labeled secondary antibody. A shift of the blue histogram to higher fluorescence intensities compared to the red histogram indicates the presence of Her2-specific antibodies. (A) Controls: plasma from mock-vaccinated mice (negative) and anti-*r*Her2 antibody (positive). (B) Plasma from vaccinated mice. Representative histograms for each Her2 vaccination group are shown.

FACS analysis of cells incubated with plasma from mice that were never exposed to the *r*Her2 antigen revealed that 3T3/neu and 3T3wt cells displayed low fluorescence intensities and that both histograms overlapped (Figure 4.24, A; Table 4.6). Conversely, incubation with the anti-*r*Her2 antibody increased the fluorescence intensity of 3T3/neu but not of 3T3wt cells, which demonstrated the functionality of the assay to detect Her2 antibodies. However, plasma from mice vaccinated with Her2 replicon-transfected DCs did not increase the fluorescence intensity of 3T3/neu cells (Figure 4.24, B; Table 4.6). The ratio of the mean fluorescence intensity (MFI) of 3T3/neu to 3T3wt cells was used to determine the relative antibody concentration (Table 4.6). Theoretically, a ratio of 1 implies that Her2 antibodies were absent in the plasma be-

cause the MFI of both cell lines was identical. In reality, mock-vaccinated mice that were not exposed to the Her2 antigen had a mean ratio of 1.9, but all other groups had a lower ratio. These data show that antibodies were not induced or induced at very low levels by the vaccination and therefore not required for the anti-tumor effect mediated by vaccination with replicon-transfected cells.

Table 4.6: Antibody assay of plasma derived from vaccinated and control mice

The mean fluorescence intensity (MFI) for 3T3wt and 3T3/neu cells incubated with plasma from individual mice (M1-M6) is shown. The relative antibody concentration is calculated by the ratio of the MFI for both cell lines. For each group, the mean of the MFI ratio is shown in the right column.

Sample		MFI (3T3wt)	MFI (3T3/neu)	MFI (3T3/neu)/ MFI (3T3wt)	mean ratio
ECD+IL-12	M1	192	187	1.0	0.8
	M2	1036	720	0.7	
	M3	667	362	0.5	
	M4	226	222	1.0	
ECD	M1	156	581	3.7	1.8
	M2	160	143	0.9	
	M3	118	178	1.5	
	M4	183	176	1.0	
IL-12	M1	276	247	0.9	1.0
	M2	381	350	0.9	
	M3	456	556	1.2	
MF+IL-12	M1	172	179	1.0	0.7
	M2	245	168	0.7	
	M3	678	284	0.4	
MF	M1	234	255	1.1	1.1
	M2	176	261	1.5	
	M3	172	189	1.1	
	M4	1364	1088	0.8	
mock	M1	170	265	1.6	1.9
	M2	71.5	160	2.2	
	M3	80.8	356	4.4	
	M4	254	250	1.0	
	M4	128	291	2.3	
	M6	452	225	0.5	
	M7	98.4	142	1.4	

4.3.4 Summary

In summary, these data show that the anti-tumor effect mediated by vaccination with Her2 replicon-transfected DCs is mediated by both major T lymphocyte subsets, CD8+ T cells as well as CD4+ T cells. Cross-priming of CD8+ T cells induced by the vaccination correlated with the anti-tumor effect of the vaccination. The role of T_H cells in mediating the vaccination effect did not involve T_H2 help for B cells to produce Her2-specific antibodies.

5 Discussion

Vaccination with BVDV replicon-transfected DCs expressing parts of the tumor antigen Her2 caused a significant anti-tumor effect. Specifically, the preventive vaccination resulted in the suppression of tumor growth and a high rate of complete protection against tumor development. The anti-tumor effect was abrogated in the absence of CD4+ and CD8+ T cells showing that both T lymphocyte subsets contributed to the anti-tumor effect. Notably, Her2-specific CD8+ T cells from vaccinated mice were responsive to cells expressing the MHC I molecule H-2^q, which corresponds to the phenotype of FVB/N mice and not to that of the injected DC2.4 cells. Hence, tumor-specific CD8+ T cells were primed by cross-presentation of the antigen. Lastly, co-administration of replicons encoding the cytokine IL-12 did not significantly enhance the anti-tumor effect, albeit a trend towards better tumor protection and stronger immune response was observed.

5.1 RNA replicons – a novel tool to load DCs for cancer immunotherapy

DC-based immunotherapy has been proven to be safer and less toxic than conventional cancer therapies in multiple phase I and II clinical trials (reviewed by Proudfoot et al., 2007 and Curigliano et al., 2007; list of trials: Mater medical research institute). Vaccines using DCs possess a higher efficacy compared to vaccination with soluble antigen or antigen-encoding nucleic acids as determined in the mouse model (Yu et al., 2008; Chan et al., 2006). Albeit this field has seen significant progress in the last years (Kalinski et al., 2009 (A)), the efficacy of DC-based cancer immunotherapy is still low in humans as exemplified by the complete failure of a phase III clinical trial in melanoma patients (Schadendorf et al., 2006). In order to improve the use of DCs in immunotherapies, parameters for a successful therapy need to be defined, including the optimal DC maturation status, the route of administration, the type of tumor antigen and, most importantly, the optimal antigen-delivery strategy (Gilboa, 2007). Notably, the antigen loading method also entails the safety of the DC-based therapy in humans.

5.1.1 Non-viral loading strategies for DCs

Several strategies for loading DCs to immunize against cancer have been evaluated in mice; many of them using Her2 as a model TAA. Ercolini et al. (2003) reported delayed tumor onset after challenge with Her2-expressing NT-2 cells in FVB/N mice after vaccination with DCs loaded with a heteroclitic variant of a Her2 peptide. Another study that applied virus-like particles from the murine polyoma virus with a fusion protein of $Her2^{1-683}$ to load DCs demonstrated complete protection against tumor

challenge with Her2-expressing D2F2/E2 cells in Balb/c mice (Tegerstedt et al., 2007). Conversely, Tegerstedt et al. showed that loading DCs with the full length Her2 protein did not protect vaccinated mice from tumor growth. DCs loaded with DNA encoding a truncated form of Her2 mediated a significant anti-tumor effect in a preventive vaccination model. Here, Balb/c mice were challenged with CT26 cells, which were stably transfected with a Her2 plasmid (Chang et al., 2004). RNA was used by Koido et al. (2000) to load DCs with the breast tumor antigen MUC1. Vaccinated mice were protected against a tumor challenge with MUC1-expressing tumor cells in wild type mice, but not in MUC1 transgenic mice.
These studies and others show that loading DCs with nucleic acids leads to efficient induction of a potent immune response and high vaccination efficacy in comparison to procedures that applied peptides and proteins (Oh et al., 2006; Nakamura et al., 2005; Metharom et al., 2005; Liao et al., 2004; Foy et al., 2001). In particular, the transfection of RNA was established as a straightforward method to load DCs (Van Tendeloo et al., 2001). Based on its transient cytoplasmic expression, genomic integration is avoided. Integration of nucleic acids into the genome potentially leads to the activation of oncogenes or inactivation of tumor suppressor genes, which induces cell transformation. Thus, RNA delivery into DCs has been demonstrated as a successful and safe application (reviewed by Pascolo, 2006). Moreover, as a major advantage, RNA production can be standardized at high purity by large scale *in vitro* transcription.

5.1.2 Viral vectors for loading DCs with tumor antigens

Besides transfecting DCs with protein-antigen, peptides or nucleic acids, the TAA can be expressed via transduction by viral vectors. For this purpose, recombinant modified viruses are applied that transduce cells with a similar mechanism as the corresponding wild type virus. Appropriate viral vectors are commonly constructed such that the formation of progenitor infectious viral particles is prohibited and that parts of the viral genome are replaced by a transgene allowing for the expression of the transgene in transduced cells. Immunotherapies based on viral vectors were shown to induce potent anti-tumor responses related to high efficiencies of transduction, the expression of high levels of antigen, and a strong adjuvant effect (reviewed by Harrop et al., 2006). Multiple viral vectors have been tested for DC-based immunotherapy against Her2 in mouse models. Thus, DCs transduced with an infectious lentiviral vector, which encoded a kinase-deficient form of Her2, protected 33% of vaccinated C57BL/6 mice against challenge with a Her2 transgenic murine prostate cancer cell line (Mossoba et al., 2008). Vaccination in a Her2 transgenic model with a retroviral vector encoding Her2 that lacked the intracellular domain resulted in significantly smaller tumors after tumor challenge and increased disease-free survival time (Nabekura et al., 2008). Finally, a very potent vaccination was established by transducing

DCs with a recombinant, replication-deficient adenovirus encoding the extracellular domain and the transmembrane sequence of Her2 (Park et al., 2008). Thus, vaccination of Balb/c mice that were challenged with Her2-expressing TUBO cells could be cured even after large tumors had established or multiple metastases had formed in the lung.

Despite of the high efficacy of vaccination strategies applying DCs transduced with modified viral vectors, the administration of viral vectors in humans is associated with serious safety concerns. The treatment with an adenoviral vector has proven to be life threatening (Marshall, 1999). Other patients suffered from the development of cancer after gene therapy with a retroviral vector (Hacein-Bey-Abina et al., 2008). The main safety concerns are insertional mutagenesis mediated by retroviral vectors and infection by a replication-competent virus. Residual infectious viral particles may remain during the production with helper viruses or helper cell lines such as shown for herpesvirus, adenovirus and alphavirus. Another major drawback represents the pre-existing immunity of the host against some vectors. Previous infection of the host by the wild type virus induces antibodies that may neutralize the viral vector. The prevalence of pre-existing immunity is especially high for poxvirus, adenovirus and herpes virus (Romano et al., 2009; Harrop et al., 2006). Accordingly, these drawbacks, in particular the safety concerns, might render the application of virus-derived antigen-delivery strategies not suitable for clinical implementations.

5.1.3 Critical evaluation of replicons as a loading tool for DC vaccination/immunostimulatory effects of BVDV replicons

In the study presented, vaccination with BVDV replicon-transfected DCs induced an antigen-specific T cell response and mediated a preventive anti-tumor effect (see Figure 4.14, Figure 4.15, Figure 4.21 and Figure 4.23). The strategy to use replicons for DC loading combines the advantages of both antigen-encoding mRNA and viral vectors. The RNA replicons are non-infectious and gene-expression does not involve integration into the host genome. Therefore, the use of replicons addresses all safety issues for the use of viral vectors in humans. Furthermore, replicons mediate high expression levels of the antigen due to amplification of the template RNA. Like viral vectors, replication of RNA constructs mimics viral infection, which, in turn, mediates an adjuvant effect. In particular, the double-stranded RNA (dsRNA) intermediates of the replication process are expected to bind to intracellular Toll-like receptors (TLR). Along this line it was shown that an alphaviral replicon activates the TLR3-dependent pathway (Diebold et al., 2009), and it is supposed that the induction of TLR3 occurs in the same way with BVDV replicons. Moreover, infections with cytopathogenic viruses were found to stimulate TLR 2, 4 and 7 expression (Werling et al. 2005). TLR signaling induces differentiation and maturation of DCs (Asselin-Paturel et al., 2005), enhances

antigen presentation on MHC II molecules (Wille-Reece et al., 2005) and increases the responsiveness to immunostimulatory cytokines (Sporri & Reis e Sousa, 2005).
Specifically, the cytopathogenic form of BVDV was demonstrated to induce the expression of the interferon regulatory factor 3 and type I interferons (IFN-α/β) (Werling et al., 2005; Adler et al., 1997; Perler et al., 2000). IFN-α/β are known to have immune-enhancing qualities (Tough, 2004; Bogdan et al., 2004). That is, the cytokines play a major role in the immediate defense against virus infection due their ability to activate the innate immune system and, thus, a fully functional adaptive immune response. Additionally, type I IFNs directly affect cells of the adaptive immune system, for instance by inducing T_H1 cytokine production (Trinchieri et al., 1996). Of note, type I IFNs have been described to enhance cross-priming (Le Bon & Tough, 2008).
Most importantly, in contrast to previously used viral vectors and RNA replicons that derived from Semliki Forest virus (Ying et al., 1999), Kunjin virus (Anraku et al., 2001), Venezuelan equine encephalitis virus (Cassetti et al., 2003) and Sindbis virus (Cheng et al., 2006), the here applied BVDV replicon originates from a non-human pathogen. Hence, pre-existing immunity against the viral antigens can be mainly excluded.
A limitation of this study is the *ex vivo* loading of DCs, which is a major restraint considering a potential application in the clinic. Thus, with each individual patient, it would be necessary to take progenitor cells (PBMCs), and to mature, load and return the DCs under high safety conditions. Consequently, such a therapy would be labor intensive and expensive. One approach to address this issue is to target DCs *in vivo*, i.e the transfection of DCs within the body. *In vivo* targeting of DCs would enable a large scale production of the vaccine and a standardization of the product quality. Furthermore, DCs retain their natural functionality and location as opposed to the artificial *ex vivo* culturing of DCs (see 5.3 Perspectives).

5.1.4 Co-delivery of immunostimulatory factors

As with all DCs, also the here-applied DC2.4 cells are known to migrate to the site of T cell priming. This fact was exploited to also deliver immunostimulatory cytokines to the DC-T-cell interaction site with the aim to further activate vaccination-induced T cells. IL-12 has been tested in several immunotherapy studies (reviewed by Del Vecchio et al., 2007), some of them using DCs to deliver IL-12 (Mazzolini et al., 2005). While a systemic administration of the cytokine is known to be toxic at higher doses (Leonard et al., 1997), the paracrine application is safe and efficient (Kang et al., 2001; Salem et al., 2004). Transfection of DC2.4 cells with Repl-IL12 indeed led to the expression and release of the cytokine as demonstrated *in vitro* (see Figure 4.12). However, the anti-tumor effect was not significantly improved. Yet, a trend towards tumor growth protection in mice that were co-vaccinated with Repl-IL12, occurred for both Her2 constructs.

These data disagree with other studies that showed a clear beneficial effect of IL-12 on the vaccination efficacy with DCs encoding a TAA (Mazzolini et al., 2005; Chen et al., 2001; Iinuma et al., 2006). It is conceivable that the additional administration of IL-12 failed to further enhance the immune response, since the adjuvant effect of the vaccine, the activation of TLRs by dsRNA, as well as danger signals induced by apoptosis, already induced IL-12 or IFN-γ secretion (Bekeredjian-Ding et al., 2006; Napolitani et al., 2005; Feng et al., 2002). Nevertheless, the data demonstrated that replicons represent reasonable tools for the expression and secretion of a cytokine. Therefore, other cytokines or co-stimulatory molecules (see 5.3 Perspectives) encoded by replicons may be used in future approaches aimed at improving the vaccination efficacy of the presented vaccination method.

5.1.5 Preventive vs. therapeutic vaccination

The anti-tumor effect of the vaccination with replicon-transfected DCs was limited to the preventive vaccination strategy. This may be due to a number of reasons that make the eradication of already established tumors a significantly bigger challenge than the prevention of tumor growth. First, the immune system faces a large number of tumor cells, i.e. tumor growth may be faster than the rate of immune-mediated killing of cancer cells (Hanson et al., 2000). Second, tumor cells become physically less accessible the larger the tumor grows (Finn, 2003 (A)). Third, the tumor has already evolved to escape the immune system, a process called immunoediting. For instance, downregulation of MHC I expression was observed in about 70%–95% of all human tumors (Garrido & Algarra, 2001; Algarra et al., 2004). Tumor-specific immune cells can also be tolerized in the tumor environment (Khong & Restifo, 2002), e.g. by the secretion of immunosuppressive cytokines in the tumor stroma. Fourth, due to the genomic instability of tumor cells, mutations are extremely common and give rise to genetically different tumor cell clones (Vogelstein et al., 2004; Dunn et al., 2004). Individual clones may become resistant to the targeted immunotherapy, which, in turn, leads to the selection of those cells and the consequent outgrow of a new tumor that is non-responsive to the treatment. A further implication of the high mutation rate of tumor cells is related to the lost dependence on one particular signaling pathway. It has been shown in a conditional mouse model of Her2 expression that primary tumors regress after de-induction of Her2 expression, but that secondary Her2-independent tumors develop (Moody et al., 2002).

So far, the success rate of therapeutic cancer immunotherapy has been poor in clinical trials (Kalinski et al., 2009 (B)). Also in mice, most of the tested regimens failed to mediate an anti-tumor effect against pre-established tumors, and therapeutic effects of cancer vaccines were found to be dependent on the time point of vaccination post tumor induction (Lollini et al., 2006). Conversely, preventive cancer immunotherapy was by far more effective and easier to achieve in mouse studies performed over the

last decade. Vaccination in the absence of a tumor allows priming of the immune system against TAA under ideal conditions, which mediates a rapid and efficient response in the event of tumor cell onset. Preventive cancer vaccines may also have applications for patients with increased risk of developing cancer resulting from a genetic pre-disposition or carcinogen exposure, as follow up treatment to surgery, chemo- or radiotherapy to target residual tumor cells, or to treat pre-cancerous lesions (Finn, 2003 (B); Quaglino et al., 2004; Astolfi et al., 2005).

5.2 Replicon-induced cross-priming of T lymphocytes contributes to the *in vivo* effect of vaccination

Vaccination with replicon-transfected DCs elicited a T cell response that protected mice effectively against tumor growth. We demonstrated the role of Her2-specific CD8+ T cells and CD4+ T cells in the depletion experiment (see Figure 4.21). Importantly, T cells recognizing the tumor antigen were primed by cross-presentation of the TAA in the experimental model where we used DCs and mice from different immunological backgrounds. In contrast to the cellular response, the humoral immune system was not activated by the vaccination (see Figure 4.24).

5.2.1 The immune response induced by vaccination

Multiple Her2 vaccination studies have already demonstrated the requirement of CD8+ T cell activation for a significant anti-tumor effect (Chen et al., 2001; Yo et al., 2007; Reilly et al., 2001; Mukai et al., 2002; Pilon et al., 2001). The importance of CD8+ T cells in cancer immunotherapy is likely caused by their direct killing ability. Furthermore, Her2-specific CD8+ T cells produce IFN-γ, which represents a proinflammatory and antiangiogenic cytokine (see 2.2.2).

In our study, depletion of CD4+ cells prior to tumor challenge abrogated the anti-tumor response mediated by preventive vaccination. Similarly, Dranoff et al. (1993) showed a CD4+/CD8+ T cell dependence in vaccination experiments with irradiated tumor cells expressing granulocyte-macrophage colony-stimulating factor by depleting either T cell subset prior to tumor challenge. Other studies have argued that CD4+ T cells are required for the priming of CD8+ T cells (Foy et al., 2001; Curcio et al., 2003). However, since the depletion in our study was performed following vaccination, this excluded a sole role of the CD4+ T cells in CD8+ T cell priming. Instead, it may be assumed that CD4+ T cells mediate other anti-tumor responses, such as immunostimulatory cytokine production or innate immune cell activation (see 2.2.2). Moreover, by releasing various apoptosis-inducing signals, T_H cells may directly kill tumor cells expressing MHC II molecules (Perez et al., 2002: Schattner et al, 1996; Thomas & Hersey, 1998; Echchakir et al., 2000). Of note, CD4+ T cell help is not only required at the priming state of vaccination (reviewed by Bevan, 2004), but it was also described to be important for the acquisition of effector functions by memory

CD8+ T cells (Gao et al, 2002; Ryu et al., 2009) or the recruitment of CD8+ T cells to the tissue harboring antigen-expressing cells (Yusuke et al., 2009).
Contradictory results have been obtained from studies evaluating the importance of antibodies for anti-tumor effects of Her2 vaccines. Several studies found an antibody-independent response against the tumor (Chen et al., 1998; Mukai et al., 2002; Pilon et al., 2001; Lindnencrona et al., 2004). Other reports described the requirement of a humoral response for an anti-tumor effect (Nanni et al., 2001; Park et al., 2008). For example, Park et al. (2008) showed that the therapeutic effect of DCs transduced with a Her2-encoding adenoviral vector was exclusively antibody-dependent. In addition, the passive administration of the humanized Her2 antibody Trastuzumab has been proven to have a therapeutic effect in patients with Her2-positive breast cancer (Hudis, 2007). Nevertheless, the overall importance of antibodies in cancer immunotherapy is a subject of debate because several tumor antigens are not located on the cell surface and therefore are not accessible to antibodies.
In summary, it has yet to be established which branches of the immune response need to be activated by an efficient cancer vaccine. Most likely, a complex interplay of various parts of the immune system is needed to prevent tumor growth or to eradicate established tumors (Chen et al., 2001; Reilly et al., 2001; Vasovic et al., 1997).

5.2.2 Cross-presentation of vaccine antigens

The study was specifically designed to evaluate the role of cross-priming. To this end, DCs/APCs (DC2.4) of the H-2^b MHC haplotype were used to vaccinate mice of the H-2^q haplotype. As a consequence, antigen-epitopes presented on MHC molecules of the vaccine APCs were not recognizable by the T cells of the vaccinated (host) mouse. Nevertheless, the fact that Her2-specific T cells were primed during vaccination revealed that these lymphocytes were primed by cross-presentation of the TAA through host APCs. In summary, this result demonstrates that cross-priming of T cells alone is sufficient to mediate a significant anti-tumor response and indicates the importance of cross-priming for vaccination strategies.
Cross-priming has been shown to play an important role for the induction of the immune response against virus-infected and transformed cells because certain viral and tumor antigens are not accessible for APCs (Jirmo et al., 2009; Heath & Carbone, 2001; Nguyen et al., 2002; Chen et al., 2004). Though direct priming of CD8+ T cells could have occurred due to infection of APCs in the case of herpes simplex virus 1, cross-priming was by far more potent in CD8+ T cell activation and it turned out sufficient to elicit an effective anti-viral response (Jirmo et al., 2009). Similarly, the CD8+ T cell response against a vaccine antigen induced by the injection with a modified vaccinia virus Ankara vector is dominated by cross-priming (Gasteiger et al., 2007). Along the same line, Cho et al. (2001) demonstrated that DNA vaccination of mice via gene

gun approaches induces both direct and cross-priming of CD8+ T cells; however, cross-priming induces a more rapid, vigorous and prolonged CD8+ T cell proliferation. Of note, antigens that enter the cross-presentation pathway are presented on MHC I as well as on MHC II molecules (Heath & Carbone, 2001). Thus, the induction of cross-priming can overcome a major drawback of viral and non-viral delivery methods for DC-based vaccinations, namely the endogenous production and exclusive presentation on MHC I molecules (Dauer et al., 2008). As mentioned earlier, both CD8+ and CD4+ T cells are important for an efficient anti-tumor response.

Methods to induce cross-priming

Considering the efficiency of cross-priming for vaccine-induced activation of the immune system, multiple studies have been pursued to evaluate strategies of targeting antigens into the cross-priming pathway (Tacken et al., 2007). For example, to achieve cross-presentation of a specific vaccine antigen, peptides bound to heat shock proteins were shown to be loaded on MHC I via the cross-presentation pathway and thereby induce CD8+ T cells and mediate clinical responses against tumor cells (reviewed by Mushid et al., 2008). Other approaches employed *in vitro* and *in vivo* loading of DCs with immune complexes (Regnault et al., 1999), virus-like particles (Bohm et al., 1995) or apoptotic cells (Akiyama et al., 2003).

Apoptosis and cross-priming

The uptake of apoptotic cells by APCs is an efficient way of providing antigens for the cross-presentation pathway (Albert et al., 1998). As already outlined above, apoptotic cell death induced by pathogens elicits danger signals like TLR ligands and uric acid that can mediate an adjuvant effect (Winau et al., 2005; Winau et al., 2006; Shi et al., 2003). Danger signals, i.e. factors associated with a dangerous situation like an infection, are required to induce an effective immune response and to avoid the induction of antigen tolerance (Gallucci & Matzinger, 2001). The adjuvant effect of these danger signals can be explained by the activation and maturation of APCs that take up dying cells and activate T lymphocytes. Importantly, APCs are simultaneously activated to antigen uptake, which further stimulates the immune response (Heit et al., 2008). Accordingly, some vaccination strategies aim at combining the adjuvant activity of apoptosis with antigen delivery to induce a strong anti-viral or anti-tumor T cell response. Methods of exploiting the mechanism of cross-priming mediated by apoptotic cells include direct vaccination with apoptotic tumor cells or induction of apoptosis in cells expressing the antigen *in vivo* (Schnurr et al., 2002). For example, Sasaki et al. (2001) significantly enhanced the priming of T cells and the anti-tumor response of a DNA vaccine by including genes encoding the influenza hemagglutinin, nucleoprotein or a mutant caspase. Chattergoon et al. (2000) transfected cells with a pENV(HIV) construct and a DNA cassette expressing Fas. The Fas-induced apoptosis

increased antigen uptake by host APCs, which led to a stronger CD8+ T cell and T_H1 response.
Another mechanism of inducing apoptosis of antigen-bearing cells is the cytopathic effect of viral vectors or virus derived replicons. Thus, it was found that the CD8+ T-cell-mediated protection of mice which had been vaccinated by intra muscular injection of replicon RNA of Semliki Forest virus was dependent on the cytopathic effect mediated by the replicon (Ying et al., 1999). Furthermore, Leitner et al. (2004) showed that the vaccination effect that was obtained with an alphavirus-based replicon was abolished when the anti-apoptotic protein Bcl-x_L prevented apoptosis.
The significance of cross-priming for the vaccination effect of BVDV replicons was first demonstrated by Racanelli et al. (2004) by comparing a non-cytopathic and a cytopathic replicon that encoded the HCV NS3 protein (see Introduction, 2.3.1). The BVDV replicon approach was assumed to be particularly suitable for efficient cross-priming due to a relatively delayed cytopathic effect of the replicating BVDV RNA. That is, apoptosis of the transfected DC was found to occur at 24 h to 48 h post transfection (see also Figure 4.9). This leaves sufficient time for the migration of the vaccine DCs/APCs to the secondary lymphoid organs, the emission of danger signals and the accumulation of a high amount of antigen, which then is cross-presented.

DCs are required for vaccination with cytopathic replicons

The here-applied vaccination procedure with heterologous DCs transfected with replicons cannot be directly compared with immunotherapies where DCs are applied for the purpose of presenting antigens and providing co-stimulatory signals to directly activate CD8+ and CD4+ T cells. Obviously, this raises the question, whether DCs are essentially needed for cross-presentation of replicon-encoded antigens, or if any other cell type may substitute for the DCs. Therefore, DCs apparently possess functions beyond antigen presentation and direct activation of T cells that are important for the vaccination effect. For example, an important characteristic of DCs may be the potential to migrate; i.e., upon activation, the cells migrate from the periphery to the secondary lymphoid organs where T lymphocytes and professional APCs reside (Randolph et al., 2005). Racanelli et al. (2004) showed that replicon-transfected DC2.4 cells can indeed be found in the draining lymph nodes as soon as 6 h post injection. Hence, the high APC concentration at these sites is likely needed to increase the effect of cross-presentation of antigens delivered by replicon-transfected DCs. Specifically, CD8α+ DCs, the most potent cross-presenting APCs, are located in the secondary lymphoid organs (Heath et al., 2004). Additionally, spleen and lymph nodes represent the locations where T lymphocytes get in contact with APCs to be activated against an encountered antigen (Abbas et al., 2007). Aside from the migratory potential of DCs, they may provide immunostimulatory cytokines such as IL-12 (Guermonprez et al., 2002) and thereby provide an adjuvant effect.

5.3 Perspectives

According to Dauer et al. (2008), the requirements for a successful DC-based immunotherapy are as follows: (i) effective *in vivo* targeting of DCs, (ii) antigen presentation on MHC I and MHC II molecules to induce an efficient T cell response and (iii) induction of DC maturation and a T_H1 polarized response to mediate an anti-tumor effect. As shown in this study, cytopathic BVDV replicons meet the second requirement for a successful vaccination by inducing both CD8+ T cell and T_H cell responses. Moreover, the replicons directly mediate an adjuvant effect through dsRNA intermediates and the induction of apoptosis. Both factors are known to induce DC maturation (Winau et al., 2005).

To also meet the first criterion, it will be necessary to develop novel, efficient *in vivo* delivery procedures for the BVDV replicons. Thus, liposomes and polymer microparticles associated with targeting moieties like antibodies or DC receptor ligands (Tacken et al., 2007) are presently tested by various laboratories for RNA delivery. A novel, interesting method for *in vivo* delivery of antigens to DCs consists of exosomes derived from DCs *in vitro* (reviewed by Mignot et al., 2006). Exosomes are secreted vesicles that contain, amongst others, cell recognition molecules on their surface that facilitate specific targeting. The vesicles have been proven to be safe for the clinical application and efficient in the delivery of antigenic peptides (Escudier et al, 2005). Furthermore, Valadie et al. (2007) demonstrated that RNA can be packaged into exosomes and subsequently transported and expressed in target cells. Thus, exosomes might be used as vectors to specifically deliver replicon RNA from transfected DCs *in vitro* to naïve DCs *in vivo*. Studies are currently underway to test the transfer of replicon RNA into exosomes.

The efficacy of a potential immunotherapy with replicon-transfected DCs may be improved by further enhancing the immune response or by combining this procedure with other treatment modalities such as chemotherapy, tumor-targeted therapies or other immunotherapy approaches. A more potent immune response may be achieved by two general mechanisms, (i) delivering immuno-stimulatory signals or (ii) inhibiting immuno-suppressive signals (Gilboa, 2004).

Immuno-stimulatory signals may include cytokines such as IL-7 or IL-15 or co-stimulatory molecules such as CD27 or OX40 (Palucka et al., 2008). As explained (section 5.1.4 Co-delivery of immunostimulatory factors), these molecules may be delivered by other replicon molecules as attempted here with IL-12.

The immune system is controlled by mechanisms that ensure homeostasis after activation. However, these inhibiting mechanisms also protect tumors against eradication by the immune system. Therefore, the abrogation of the immunosuppressive tumor environment exposes cancer cells to the immune effector mechanisms induced by immunotherapy. Neutralizing antibodies or agonists against suppressive cytokines like

IL-10, IL-13 or TGF-β have been used to overcome an immunosuppressive milieu (Moore et al., 2001; Terabe et al., 2000; Li et al., 2006). Alternatively, the depletion of regulatory T cells resulted in increased anti-tumor immunity in mice (Onizuka et al., 1999). The elimination of immunosuppressive T cells in humans can be achieved by certain chemotherapeutic drugs like cyclophosphamide (Bass & Mastrangelo, 1999), demonstrating a potential synergistic effect of the combination of immunotherapy and chemotherapy. Accordingly, Cuadros et al. (2003) significantly improved the anti-tumor effect of DC-based Her2-targeted immunotherapy by co-administering an anti-angiogenic factor. Furthermore, it is conceivable to combine two different immunotherapy regimens to significantly improve vaccination efficacy. For instance, Wolpoe et al. (2003) combined a Her2-targeted whole tumor cell vaccine with therapeutic antibodies. In view of the scenario that the vaccination with replicon-transfected DCs elicited a strong T cell response but no antibodies against Her2 (see Fig. Figure 4.24), a combination with the already established therapeutic Her2-antibody Trastuzumab might have a synergistic effect and may enhance the vaccination efficacy against Her2-expressing tumors.

5.4 Conclusion

The transfection of DCs with cytopathic BVDV replicons was established here as a novel approach for DC-based cancer immunotherapy. As a particular advantage, BVDV replicons combine the safety of an mRNA antigen delivery method with the adjuvant effect of viral vectors. Vaccination with BVDV replicons induced a potent preventive anti-tumor vaccination that was shown to be exclusively mediated by cross-primed T lymphocytes. Accordingly, this study particularly highlights the potential of cross-priming to induce a potent immune response in cancer immunotherapy. The obtained data further demonstrate that the anti-viral response mediated by vaccination with BVDV replicons (Racanelli et al., 2004) could be translated in an anti-tumor response. This indicates that similar vaccination strategies are applicable for both, virus-infected and transformed cells.

Future studies should apply BVDV replicons expressing the Her2 antigen to *in vivo* transfection of DCs. Moreover, combinations with other therapies such as with therapeutic monoclonal antibodies directed against Her2 should be evaluated to enhance the anti-tumor effect and to even enable the eradication of pre-existing tumors.

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7 Lists

7.1 Abbreviations

AG	antigen
APC	antigen presenting cell
bp	base pairs
BVDV	Bovine Viral Diarrhea Virus
CD	cluster of differentiation
CPE	cytopathic effect
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
ECD	extracellular domain
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot
EMA	ethidium monoazide
EMCV	Encephalomyocarditis virus
FACS	fluorescence-activated cell sorting
FSC-A	forward scatter-area
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HCV	hepatits C virus
Her2 (*h*/*r*)	human epidermal growth factor receptor 2 (human/rat)
i.p.	intraperitoneal
ICD	intracellular domain
IFN	interferon
IFN-γ	interferon γ
IgG	immunoglobulin G
IL	interleukin
IRES	internal ribosome entry site
MF	middle fragment

MFI	mean fluorescence intensity
MHC	major histocompatibility complex
(*m*)IL-12	(murine) interleukin 12
miRNA	microRNA
mRNA	messenger RNA
MUC1	mucin 1
NS	non-structural protein
ns	not significant
OLP	overlapping peptides
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phyco-erythrin
RE	restriction enzyme
Repl-hECD/-hMF/-hICD/-rECD/-rMF/-rICD/-IL12	bi-cistronic BVDV replicon encoding hECD/hMF/hICD/rECD/rMF/rICD/mIL-12
RNA	ribonucleic acid
s.c.	subcutaneous
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SFU	spot forming unit
siRNA	small interfering RNA
SSC-A	side scatter-area
TA	tumor antigen
TAA	tumor associated antigen
TCR	T cell receptor
TGF-β	transforming growth factor β
T_H	T helper cell
TLR	Toll-like receptor
TM	transmembrane domain
T_{reg}	regulatory T cell
UTR	untranslated region

7.2 Figures

7.3 Tables

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Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit selbstständig verfasst und keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet wurden. Die wissenschaftliche Arbeit wurde an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht.

Washington, DC, January 2010 Birgit Edlich