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INCREASED EFFICACY OF Fc RECEPTOR-TARGETED CANCER VACCINES

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CONTENTS

I. ABBREVIATIONS

ACT – adoptive cell transfer

APC - antigen presenting cell

ATCC - American Type Culture Collection

BM – bone marrow

BSA - bovine serum albumin

CEA – carcinoembryonic antigen

CTL - cytotoxic T lymphocyte

DC - dendritic cell

EGF – endothelial growth factor

EGFP - enhanced green fluorescence protein

FcγR – Fcgamma receptor

GD2 - GD2 ganglioside

GM-CSF - granulocyte-macrophage colony-stimulating factor

HRP - horseradish peroxidase

IL – interleukin

i.m. - intramuscular

i.p. - intraperitoneally

i.v. - intravenous

INF – interferon

KLH - keyhole limpet hemocyanin

mAb - monoclonal antibody

MFI - mean fluorescence intensity

MHC - major histocompatibility complex

NK – natural killer

OD – optical density

PE - phycoerythrin

PFU – plaque forming unit

PSA - prostate-specific antigen

s.c. – subcutaneous

TAA - tumor associated antigen

TBI – total body irradiation

TCR - T-cell receptor

Th1 - T helper type 1

Th2 - T helper type 2

TILs – tumor infiltrating lymphocytes

TK – thymidine kinase

TLR – toll like receptor

TNF – tumor necrosis factor

Tregs – T regulatory cells

TSA – tumor specific antigen

VEGF – vascular endothelial growth factor

VGF – vaccinia growth factor

WT - wild type

rOVV – recombinant oncolytic vaccinia virus

II. ABSTRACT

A major challenge for inducing antitumor immune responses with native or modified tumor/self-antigens in tumor-bearing hosts relates to achieving efficient uptake and processing by dendritic cells (DCs) to activate immune effector cells and limit the generation of regulatory T-cell (Treg) activity. In my experimental work I have analyzed the ability of therapeutic DC vaccines expressing a CD166 cross-reactive mimotope of GD2 ganglioside, 47-LDA, to selectively expand adoptively transferred tumor-specific T cells in NXS2 neuroblastoma tumor-bearing syngeneic mice. Prior to the adoptive cell transfer (ACT) and DC vaccination, the tumor-bearing mice were lymphodepleted by nonmyeloablative total body irradiation (TBI) or a myeloablative regimen that required bone marrow (BM) transplantation. The 47-LDA mimotope was presented to DCs either as a linear polypeptide in conjunction with universal T helper epitopes or as a fusion protein with the murine IgG2a Fc fragment (47-LDA-Fcγ2a) to deliver the antigenic cassette to the activating Fcgamma receptors (FcγRs). It was observed that immunization of adoptively transferred T cells with the 47-LDA mimotope expressed in the context of the activating Fc fusion protein induced higher levels of antitumor immune responses and protection from primary tumor growth than the 47-LDA polypeptide DC vaccine. Moreover, when the antigen-experienced or naïve T cells were injected together with the DC vaccines to lymphodepleted mice whose primary tumor was surgically excised, the 47-LDA-Fcγ2a vaccine-induced T cells were more protective against spontaneous metastases than those induced by the 47-LDA polypeptide-coated DC. The highest antitumor efficacy of 47-LDA-Fcγ2a vaccine-generated T cells was detected in myeloablated mice and was associated with enhanced expansion of functional cytotoxic T cells and reduced generation of immunosuppressive Treg cells. It was also observed that the antitumor efficacy of the therapeutic 47-LDA-Fcγ2a vaccine was comparable to that achieved by a virotherapy-associated cancer vaccine using a recombinant oncolytic vaccinia virus (rOVV) expressing the 47-LDA-Fcγ2a fusion protein. The latter treatment however did not require TBI or ACT and resulted in induction of antitumor immune responses in the setting of established tolerance, paving the way for testing novel anticancer treatment strategies.

In summary, this work highlights a new application of peptide mimotopes expressed in the context of the activating Fc fusion protein for tuning of DC responses

to counteract the immunosuppression associated with tumor progression during therapeutic vaccination. It stresses the importance of exploring the uptake and processing of tumor/self-antigens by DCs to activate immune effector cells and limit the ability of attracting anti-inflammatory Treg cells. These findings illuminate a new paradigm for cancer immunotherapies aimed at the selective activation of the inflammatory versus regulatory type of immune responses.

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III. STRESZCZENIE

„Zaangażowanie receptorów Fc zwiększa efektywność szczepionek nowotworowych”

Kluczowym elementem immunoterapii nowotworu z wykorzystaniem szczepionek opartych na natywnych lub zmodyfikowanych antygenach rakowych jest uzyskanie efektywnego pobierania i procesowania antygenów przez komórki dendrytyczne [ang. dendritic cells (DCs)]. Proces ten ma prowadzić do aktywacji komórek efektorowych jednocześnie ograniczając indukowanie komórek regulatorowych [ang. T regulatory cells (Tregs)]. W mojej pracy badawczej analizowałam zdolność szczepionki terapeutycznej DC do wywołania ekspansji przeszczepionych swoistych limfocytów T, które rozpoznawały komórki neuroblastoma NXS2 w syngenicznym modelu mysim. DCs prezentowały mimikę gangliozydu GD2 (czyli peptyd naśladujący ten gangliozyd), 47-LDA, która wywoływała reakcje krzyżowe z białkiem CD166. Przed infuzją specyficznych dla komórek NXS2 limfocytów T oraz immunizacją, myszy A/J z wyindukowanym guzem NXS2 były poddane niemieloablacyjnemu napromieniowaniu całego ciała [ang. total body irradiation (TBI)] lub mieloablacyjnemu kondyncjonowaniu, które wymagało przeszczepu szpiku kostnego. DCs prezentowały peptyd 47-LDA, zawierający również dwa uniwersalne peptydy pomocnicze [ang. T helper peptides (Th)], w postaci liniowej lub jako białko fuzyjne zawierające mysi fragment Fc przeciwciała IgG2a (47-LDA-Fcγ2a). Połączenie peptydu 47-LDA z częścią Fc miało na celu dostarczenie antygenu do DCs poprzez aktywujący receptor gamma [ang. Fcgamma receptors (FcγRs)]. Zaobserwowano, że immunizacja przeszczepionych limfocytów T szczepionką 47-LDA-Fcγ2a-DC prowadziła do indukcji silniejszej odpowiedzi przeciwnowotworowej i protekcji przed wzrostem guza niż szczepienie z wykorzystaniem DCs prezentujących polipeptyd 47-LDA. Dodatkowo, antygenowo-swoiste lub naiwne limfocyty T podane wraz ze szczepionką 47-LDA-Fcγ2a-DC były bardziej skuteczne w zapobieganiu spontanicznym przerzutom u myszy, którym chirurgicznie usunięto guz, niż limfocyty T uczulane DCs opłaszczonymi polipeptydem 47-LDA. Najsilniejszy efekt przeciwnowotworowy szczepionki 47-LDA-Fcγ2a-DC, związany ze wzmożoną ekspansją limfocytów T cytotoksycznych i zredukowaną indukcją komórek Treg, zaobserwowano u myszy

poddanej całkowitej mieloablacji. Równocześnie, porównywalny poziom protekcji uzyskano poprzez zastosowanie wirusowej terapii przeciwnowotworowej wykorzystującej zrekombinowany, onkolityczny wirus krowianki [ang. recombinant oncolytic vaccinia virus (rOVV)] wykazujący ekspresję białka fuzyjnego 47-LDA-Fcγ2a. Wirusoterapia indukowała odpowiedź przeciwnowotworową w stanie tolerancji, nie wymagając zastosowania TBI ani immunoterapii adoptywnej.

Podsumowując, mimiki peptydowe w połączeniu z częścią Fc przeciwciała stanowią nową metodę modulowania aktywności DCs podczas immunizacji, przeciwdziałającą immunosupresji towarzyszącej wzrostowi nowotworu. Poniższa praca podkreśla znaczenie badań analizujących pobieranie i procesowanie antygenów nowotworowych przez DCs prowadzące do aktywacji komórek efektorowych i ograniczające indukcję komórek Treg. Uzyskane rezultaty stanowią część nowego modelu immunoterapii nowotworu ukierunkowanego na selektywną aktywację odpowiedzi immunologicznej i stłumienie reakcji immunosupresji.

IV. INTRODUCTION

1. Immune system as a means to fight cancer

Cancer is a leading cause of death with increasing incidence and mortality due to growth of the aging population. Despite an overall progress in cancer therapy, substantial groups of patients lack effective treatment options. Conventional strategies (e.g., surgery, radiation and chemotherapies) are often highly active in eliminating the major tumor mass, but are less effective in eradicating residual cancer cells and in preventing disease recurrence. These limitations of current treatments provide the rationale for the utilization of alternative methods in order to identify and destroy cancer cells. Among them, immunotherapy is considered as a promising approach that provides the ability to selectively eliminate transformed cells while leaving healthy tissue intact.

The idea of exploiting the immune system to eradicate disease has had a long currency in cancer research. The view that cancer could be treated by active immunization arose in the 1890s with the proposals of William Coley [1] who reported that post-surgical wound erysipelas (an infection with *Streptoccocus* spp.) induced occasional remission of cancer. However, attempts by Coley and his colleagues to stimulate systemic immunity directed against tumors with bacterial extracts met with a limited success, leading to the lost of interest in this type of approach. The modern era of tumor immunology began several decades later. The first reports suggesting existence of tumor-specific immunity were associated with the occasional spontaneous regressions of cancers in immunocompetent hosts [2]. Furthermore, the studies with mice which recovered from successfully implanted tumors and usually resisted reinoculation with the same neoplasm additionally supported this idea [3]. The development of inbred strains of mice enabled investigators to examine whether immune system was able to distinguish tumors arising in such animals from normal tissue in the same strain. The demonstration that inbred mice immunized against carcinogen-induced tumors rejected a subsequent challenge with the same tumor cells whereas did not respond to the corresponding nontransformed cells documented existence of tumor-specific immune responses [4-6]. Soon after, Burnet and Thomas introduced the concept of cancer immunosurveillance: the immunological resistance of the host against the development of cancer [7]. This idea has been challenged for

decades because of the lack of direct experimental evidence (reviewed in ref. [8]). Moreover, the immune-surveillance hypothesis was discredited when no differences in primary tumor development were found between athymic nude mice and syngeneic wild-type mice [9]. It was expected that the hosts with impaired immune system would display higher cancer incidents compare to the immunocompetent counterparts. Although nude mice developed more frequently virus-induced tumors, number of spontaneous, nonviral tumors was similar [10, 11]. The importance of immune system in controlling of tumor growth was additionally questioned when naturally induced tumors in rodents were found to be largely non-immunogenic in contrast to those induced by carcinogens [12]. Altogether, these observations contributed to the notion that cancer cells may not be sufficiently distinct from normal tissue as they do not encode tumor antigens, suggesting that the immunological intervention in cancer would not produce satisfactory results.

The turning point in cancer immunology is associated with studies of van Pel and Boon, who demonstrated that induction of protective immunity against non-immunogenic tumors was feasible, providing that tumor cells used for immunization were mutagenized by radiation or chemical agents [13]. These findings were confirmed by subsequent studies in different animal systems, including models in which immunization led to rejection of pre-existing tumors. It was demonstrated that lack of tumor immunogenicity results not from the absence of tumor-rejection antigens but from the inability of growing tumor to effectively activate immune system [14]. Furthermore, renewed interest in cancer immunosurveillance was noticed when studies on nude mice revealed that they are not totally immunocompromised as they still have nonthymic-dependent T cell population and an innate immune system [15, 16]. Consequently, severely immunocompromised, $Rag^{-/-}$ (lacking T and B cells) and $STAT^{-/-}$ (lacking interferon-mediated pathways) mice that are characterized by deficiencies in both innate and adaptive immune response, have been shown to have a significant increase in the incidences of tumors [14, 17, 18]. Importantly, all these tumors when transplanted to immunocompetent mice were rejected, suggesting that their development in the original host resulted from defects in immune surveillance.

After a century of debate as to whether the immune system can actually target tumors, a growing body of evidence now indicates that immune cells can be utilized to defeat cancer [14, 19]. Histopathology of tumor section has revealed infiltrating lymphocytes around the tumor bed and subsequent studies demonstrated that these

lymphocytes could recognize tumor antigens [20] and display tumor-specific cytotoxic activity *in vitro* [21-23]. In addition, the presence of tumor-infiltrating lymphocytes (TILs) was shown to correlate with clinical outcome in vertical-growth-phase melanoma and in breast, prostate, esophageal and colorectal carcinomas [24-28]. However, it is also known that these spontaneously arising responses against cancer are relatively ineffective [9]. This is due, in part, to the poor immunogenicity of tumor antigens which are unable to elicit strong antitumor immunity. Moreover, immunosuppressive character of tumor microenvironment and a large number of regulatory pathways appear to prevent tumor-reactive cells from generating effective immune response [8, 29]. Thus, the proper method to activate immune system against cancer cells while antagonizing the regulatory elements is the key factor in successful immunotherapy that might lead to final elimination of transformed cells.

2. The immunogenicity of tumor cells

The identification of tumor-rejection antigens and understanding the tumor-cell recognition and destruction has been a major advance in tumor immunology in the past decade. Multiple studies in experimental animals showed that both humoral and cellular immune responses were responsible for the rejection of solid tumors [30]. Thus, significant effort has been devoted towards the identification of antigens recognized by antibodies, cytotoxic T lymphocytes (CTL) as well as $CD4^+$ T helper cells. To date, approximately 70 MHC class I– and class II–associated tumor antigens have been discovered, while more than 1,700 have been identified by antibodies in cancer patients (reviewed in ref. [31]).

Based on qualitative differences, tumor antigens have been divided into two groups: tumor-specific antigens (TSAs) that are caused by mutations and are exclusively expressed by cancer cells, and tumor-associated antigens (TAAs) that result from over- or aberrant expression of non-mutated proteins. A single point mutation may activate an oncogene or inactivate tumor suppressor genes causing increased signal transduction and uncontrolled cell division. These mutations result in proteins that are expressed only by transformed cells, are involved in the induction of malignancy, and may be important in maintaining the malignant phenotype. Thus, these unique antigens can serve as compelling targets for immunotherapy and can be recognized by immune

system as non-self determinants, which results in induction of high-affinity T cells and overcomes the problem of neonatal and peripheral tolerance. The first reported human TSA was a mutated cell cycle regulator CDK4 [32]. The other well characterized tumor-specific antigens include tumor-specific chimeric fusion protein (Bcr-abl) [33], mutated oncogene-encoded proteins (ras) [34] and mutated tumor suppressor gene (p53) [35]. TSAs appear as ideal targets for immunotherapy, however, generating vaccines against those antigens is highly problematic as TSAs are unique to a specific patient's tumor and it is necessary to develop personalized therapy for individual patients. Therefore, current immune strategies mostly utilize the TAAs which are expressed by many types of cancer cells with only a limited expression on normal tissues. The TAAs can be subdivided into four major categories based on expression pattern: cancer-testis antigens (NY-ESO-1), differentiation antigens (gp100, MART-1), oncofetal antigens (alpha fetoprotein) and over-expressed antigens (PSA, wild-type p53, Her2/Neu). Peptides and proteins derived from these antigens are under intensive studies to determine their potential in inducing specific antitumor immunity**.** When melanoma-associated antigen genes (MAGE) peptides have been applied for clinical trials as cancer vaccines, clinical responses in form of tumor shrinkage and regression have been observed in a significant proportion of patients [36, 37]. The efficacy of immunotherapy targeting TAAs depends on its ability to break the immunologic tolerance that results from the abundant expression of these antigens in non-malignant tissues. However, eliciting strong immune responses towards TAAs may lead not only to tumor disruption but also to destruction of normal tissue. Several studies have demonstrated that autoimmune disease might be an inevitable consequence of effective antitumor immunity [38-41], though in some cases it is induced only in the presence of the second inflammatory agent that is introduced into the normal tissue [42, 43]. Moreover, recent studies indicate that tumor cells differ in their susceptibility to the effector arm of an immune response from their normal counterparts even if they express the same antigen [44]. Thus, immunotherapy targeting TAAs that is capable of overcoming tolerance may become a part of successful cancer treatment.

3. Carbohydrate antigens and their mimotopes in immunotherapy of cancer

Altered glycosylation is one of the hallmarks of cancer cells. Over- or under-expression of sugar moieties in glycoproteins and glycolipds occur during tumor progression, invasion and metastasis [45, 46]. The products of aberrant glycosylation, tumor-associated carbohydrate antigens (TACAs), have been found in all examined tumor cells and their presence influence the prognosis and survival of cancer patients in a manner that is proportional to the degree of expression [47]. The most common type of altered glycosylation results from enhanced expression of glucosaminyl transferase-V (GnT-V) which leads to an increase in complex, highly 1,6-branched N-glycans [47]. Other modifications include reduced O-glycosylation [48], overexpression of lacto-series type 1 and type 2 structures [49] and accumulation of ganglio- and globo-structures [50].

Tumor-expressed carbohydrate antigens are considered important targets for passive and active specific immunotherapy as they are broadly expressed in several malignancies and are associated with faster clonal growth and metastatic potential of tumor cells [50]. However, carbohydrates are typically poorly immunogenic and usually induce short-lived IgM-type antibodies in vaccinated hosts. Furthermore, most carbohydrate antigens are T-cell independent which reflects their inability to stimulate MHC class II-dependent T cell help. As a consequence, carbohydrate antigens are not effective in inducing a sufficient anamnestic immune response. For this reason, strategies have been developed to convert carbohydrates to T cell-dependent antigens, including carbohydrate-protein conjugates, anti-idiotypes and peptides resembling a carbohydrate structure as surrogate antigens.

Conjugate vaccines, in which the carbohydrate antigen is covalently attached to an immunogeneic carrier protein, are able to overcome the lack of T lymphocyte help and can induce higher titer of IgM antibodies and partial class-switching to IgG antibodies [51]. Although carbohydrate-protein conjugates such as keyhole limpet hemocyanin (KLH) show promise for active specific immunotherapy, difficulty in antigen purification or synthesis, variability in patients' antibody responses and low levels of IgG antibodies in many patients immunized with carbohydrate conjugates represent problems of this type of approach. Another strategy for enhancing carbohydrate immunity is the potential use of anti-idiotypic antibodies. This approach requires that a polypeptide immunoglobulin variable region mimics a carbohydrate

determinant, providing a surrogate immunogen with capacity to induce increased titers following booster immunization. Studies on a network manipulation suggest that in tumor-bearing hosts, the concomitant idiotypic network response controls tumor growth and progression, though the molecular basis for induction of tumor-specific immune responses by antibodies that mimic carbohydrate tumor-associated antigens has not been well elucidated. Therefore, as an additional alternative, surrogate peptide antigens that mimic carbohydrate epitopes have been developed and examined for their ability to elicit immune responses that cross-react with carbohydrate structures. Peptide mimitopes are generated by screening phage display peptide library with monoclonal antibody that was raised against a carbohydrate antigen. The isolated peptide structurally mimics the parental antigen and can break tolerance toward carbohydrate self-antigens by stimulating T cells that have not been eliminated during ontogeny due to the low affinity for the nominal antigen [52, 53].

Mimicking peptides represent a very promising tool to overcome T cell-independence and may have significant advantage as vaccines compared with carbohydrate-protein conjugates or anti-idiotypic antibodies. First, the chemical composition and purity of synthesized peptides can be precisely defined, and immunogenicity significantly enhanced by polymerization. Moreover, peptide synthesis may be more practical than synthesis of carbohydrate-protein conjugates or the production of anti-idiotypes. Furthermore, peptides can functionally emulate similar structures of TACAs, inducing antibodies that recognize multiple TACAs, and therefore, functioning as a TACA multivalent vaccine [52]. It is also anticipated that by virtue of peptides being intrinsically T-cell antigens, peptide mimotope immunization could be manipulated to lead to carbohydrate-reactive cellular responses [53]. Finally, peptide mimotopes can also be engineered into plasmids for genetic vaccination alone or in combination with genetic adjuvants to increase the level and persistence of carbohydrate-specific immune responses [54]. So far several peptide mimotopes have been described, including mimotope of neolactoseries structure Lewis Y (LeY) [55], sialylated Lewis a/x [56], GD2 [57, 58] and GD3 [59]. Immunization with carbohydrate mimicking peptides was shown not only to augment tumor-specific humoral responses [55] but also induced cytotoxic T lymphocytes [60, 61]. The activation of CTL responses by immunization with peptide mimics is of a great importance as most of carbohydrate-base vaccines are unable to elicit T cell immunity and consequently, they are ineffective in eradication of well-established cancers. The mechanisms underlying

the phenomenon of molecular mimicry between the carbohydrate antigens and their mimotopes are poorly understood. In one report, it has been shown that a peptide surrogate of O-β-linked N-acetylglucosamine activates crossreactive CTLs that recognize a processed O-linked glycopeptides associated with MHC class I molecules [62]. Other investigators have demonstrated induction of carbohydrate-specific unrestricted CTL responses with MHC class I-binding carrier peptides [63]. In addition, recent reports indicate that mimotope-based peptide vaccines may also activate CTLs that recognize cross-reactive peptide epitope expressed by other molecules on tumor cells, highlighting a new application of peptide mimotopes for immune therapy of cancer [64].

In summary, mimicking peptides may revolutionize cancer vaccines as they are efficient in inducing immune responses against carbohydrate antigens expressed by variety of tumor cells. Their efficacy results from the ability to break the tolerance towards carbohydrate self-antigens and overcome the T-cell independent nature of carbohydrates. Peptide mimetics are also likely to have advantages over carbohydrate immunogens because of the greater efficiency of manufacturing protein rather than carbohydrate compounds for vaccination. Although promising, the further investigation is required to identify mechanisms by which certain peptides function as mimotopes of carbohydrate antigens with the ability to stimulate protective or therapeutic immunity.

4. GD2 ganglioside as therapeutic target for cancer therapy

Gangliosides, sialic acid-containing glycosphingolipids, have engendered great interest in the search for molecules of relevance for tumor growth and formation of metastases and as potential targets for immunotherapy. In general, gangliosides consist of hydrophilic (carbohydrate) and hydrophobic (ceramid) portion and are expressed on the cell surface membranes of most mammalian cells. They exert diverse biological functions including cell growth regulation, cell-cell and cell-matrix adhesion and modulation of the immune system. Also, they exhibit quantitative and structural variability in cancer cells due to the changes in activity of enzymes responsible for their synthesis [65]. Several studies have demonstrated that the enhanced level of gangliosides on tumor cells was associated with higher cellular proliferation and increased metastatic potential [66, 67]. Although almost all tumors exhibit an aberrant

ganglioside pattern, the increased level of gangliosides has been reported in tumors of neuroectodermal origin, such as melanoma, neuroblastoma, glioblastoma or astrocytoma [68]. Because the differences in gangliosides expression between normal and malignant cells can be recognized by immune system, several gangliosides have been explored as targets in immunotherapy of cancer. To date, several clinical trials have investigated passive immunotherapy using murine or murine/human chimeric monoclonal anti-ganglioside antibodies in their native form or combined with various effector molecules [69-71]. Moreover, the vaccination strategy using native or structurally modified tumor-associated gangliosides in combination with adjuvants is currently under investigation in clinical studies.

There is a variety of gangliosides that represent a potent targets for immune recognition and attack including GM2, fucosyl-GM1, GD3 and GD2. Among them, the GD2 ganglioside appears as an especially attractive one. GD2 is highly expressed on neuroectodermal tumor cells [72, 73] whereas its distribution in normal tissues is restricted to neurons, skin melanocytes and peripheral pain fibers [74, 75]. Furthermore, the expression of GD2 persists on tumor cells during therapy with anti-GD2 mAb [76]. For many years, GD2 has been considered to be a marker of neuroectoderm-derived human cancers without specific biological function in the malignant properties of human cancer cells. Co-localization of GD2 ganglioside at the adhesion plaques of melanoma cells and its functional involvement in a cell adhesion process mediated by the interaction between integrins and the extracellular matrix was first reported by Cheresh et al., [77]. More recent studies revealed that GD2, integrin β1 and focal adhesion kinase (FAK) form a molecular complex across the plasma membrane, and antibody binding to GD2 induces conformational changes in integrin molecules leading to dephosphorylation of FAK and apoptosis associated with disruption of cell-matrix interaction, known as anoikis [78].

A number of therapeutic trials with mAbs specific for GD2 ganglioside have been reported and shown partial *in vivo* anti-tumor effects in patients with neuroblastoma and malignant melanoma [79-81]. The first clinical study with murine anti-GD2 mAb 14G2a injected to six neuroblastoma patients resulted in two complete remission and two partial responses [82]. Passive immunotherapy with another murine GD2-specific 3F8 mAb led to prolonged survival in children with stage IV neuroblastoma [83, 84]. The subsequent studies have revealed that the clinical response to the 3F8 mAb was associated with induction of an idiotype network which included

anti-anti-id (Ab3) and anti-GD2 (Ab3') antibodies [85]. The addition of GM-CSF to 3F8 mAb treatment has led to increased responses in patients with reflactory neuroblastoma. In a recent analysis of 27 patients with bone marrow (BM) metastasis as the only evidence of refractory disease, 85 % achieved complete recovery after therapy with 3F8 mAb combined with GM-CSF [86].

Another approach targeting GD2 ganglioside includes active immunization with cancer vaccines. This strategy was shown to elicit an antibody response in both experimental models and clinical settings [68]. However, the potential difficulty associated with active immunization resides in the induction of relatively low levels of GD2-specific IgG antibody responses [87], raising the possibility that the GD2 conjugate may not be sufficiently immunogenic in immunosuppressed individuals [88]. Several strategies have been proposed to augment immunogenicity of GD2 including conjugation to a protein carrier, immunization with anti-idiotypic antibodies or with a lactone form of GD2 ganglioside [89]. Immunization with GD2-KLH conjugates resulted in induction of IgG responses in patients with malignant melanoma [89]. The administration of TriGem^{TM}, an anti-idiotypic antibody that bears the internal image of GD2, led to generation of robust and specific IgG immune responses against GD2, with only a minimal toxicity [90]. Other investigators in a search for a method of generating a vaccine to GD2 ganglioside that is T cell-dependent and highly immunogenic focused on the GD2 mimetic peptides as surrogate antigen [57]. This latter approach resulted in induction of humoral and cellular immune responses leading to the generation of effective protection against neuroblastoma challenge in a prophylactic model [57, 61].

5. Vaccine strategies for the immunotherapy of cancer

Cancer vaccines can consist of tumor cells or recombinant TAAs [91]. First cancer vaccines involved autologous or allogeneic whole tumor cells, irradiated or otherwise inactivated, or cancer cell extracts (lysates, membranes and heat shock proteins) (reviewed in ref. [92]). Those vaccines were often combined with bacterial adjuvants in an attempt to amplify tumor-specific immune responses [93]. Subsequently, genetically modified cancer vaccines have begun to replace the complex mixtures of tumor cells. As such, tumor cells were engineered to secrete numbers of different cytokines or were transfected with genes encoding costimulatory molecules [94-98]. Of the cytokines

studies, granulocyte-macrophage colony-stimulating factor (GM-CSF) appeared most effective as it causes recruitment of antigen presenting cells (APCs) required for activation of $CD8^+$ and $CD4^+$ T cells. In addition, several of other genetically altered tumor cell vaccines expressing IL-2, IL-4, B7.1 are currently under investigation in clinical studies [99, 100]. However, despite the initial success of cell-based vaccines, the main limitation associated with this approach is the difficulty in generating tumor vaccines on a large scale. It is a highly individualized, expensive and labor intensive process. Moreover, the potential for causing autoimmunity may further limit the usefulness of this approach. Several studies have demonstrated that in addition to presenting tumor-specific antigens, activated DCs may prime immunity to many other self-antigens that are presented on cancer cells, leading to destruction of healthy tissue [101]. Thus, in order to induce more tumor-specific immune responses the emphasis has been shifted to vaccines expressing TAAs.

Understanding how T cells recognize antigens in the form of short peptides presented by MHC molecules and knowledge of the amino acid sequence of TAAs provided the basis for antigen-specific vaccination and prompted the use of peptides as therapeutic agents in the treatment of cancer. Peptides vaccines are easy to synthesize and standardize to meet the requirements for clinical trials. Consequently, they have been broadly used in the treatment of melanoma, colon, breast, ovarian, pancreatic and cervical malignancies. In general, clinical responses elicited by peptide immunization were observed in 10%-30% of the treated melanoma patients (reviewed in ref. [102]), suggesting that novel strategies must be explored to augment the vaccine efficacy. One of the approaches to increase peptides immunogenicity is epitope enhancement which involves modifications of the amino acid sequence of desire epitope so that its affinity to MHC molecules and triggering of T cell receptors (TCR) increases. When this strategy was applied to melanoma antigen gp100 peptide, 10 in 11 patients exhibited strong cellular responses [36] whereas naïve gp100 peptide resulted only in low levels of T cell responses in two of eight patients. Furthermore, peptide immunization can be combined with cytokine treatment such as IL-2 [103] or GM-CSF [104], or with the blockage of negative regulatory molecules such as CTLA-4 [105]. Based on the knowledge that CTL are induced more efficiently by professional presenting cells such as DCs, the strategy with *ex vivo* generated DCs pulsed with a peptide of interest have also been investigated. This method was shown to mediate tumor regression in mice [92] as well as generate a frequent and strong CTL responses in humans [106]. In

addition, peptides converted into DNA vaccines have been shown to provide some degree of systemic tumor protection [107] and recombinant viruses have recently been used as vectors for peptide vaccines. A number of trials utilizing vacinia virus (VV) or avipox virus expressing prostate-specific antigen (PSA) or carcinoembryonic antigen (CEA) have been reported or are in progress [108, 109]. The potency of these vectors may also be enhanced by addition of genes for immunostimulatory molecules or cytokines.

Connectively, tumor-specific vaccines in a form of short peptides, their mimotopes or altered TAAs, delivered by means of DCs, recombinant viruses or as a naked DNA, appear as a feasible, effective strategy that is capable of inducing specific antitumor immune responses. The optimal development of antigen-specific vaccines is now under investigation with a focus on identification of the most potent tumor rejection antigens and the appropriate route or vehicle by which the antigen is delivered to the immune system.

6. Adoptive cell transfer (ACT) in cancer therapy

Shortly after the demonstration that cellular arm of the immune system plays a central role in tumor rejection [110], attempts have been made to treat established tumor by a transfer of immune cells. Currently, adoptive transfer of tumor antigen-specific T cells after their *ex vivo* activation and expansion represents a promising immunotherapy approach. The first ACT studies in which transplanted syngeneic tumors were treated with splenocytes from immunized mice were concentrated on defining the characteristics of transferred T cells that influenced treatment effectiveness. As a result, $CD8^+$ T cells were showed to be required for effective antitumor effects [111] and several lines of evidence showed a correlation between the number of adoptively transferred T cells and antitumor responses *in vivo* [112, 113]. Additionally, new findings also emphasize that the quality of transferred T cells may be of importance [114]. It has been demonstrated that less-differentiated, central-memory-like T cells have a high proliferative potential and are less prone to apoptosis than more differentiated cells, suggesting that these cells might be optimal for ACT-based immunotherapy [114]. Consistent with these findings, increased antitumor responses

were observed after adoptive transfer of low numbers of early effector T cells compared with high numbers of “unfit” differentiated T cells [115, 116].

Because the presence of TILs capable of recognition of tumor antigens is well documented in cancer patients [23], the infusion of isolated autologus TILs after *ex vivo* activation was expected to become an effective treatment in cancer therapy. Indeed, early attempts of ACT treatment using autologus TILs plus high dose of IL-2 resulted in objective response rate of 34% in 86 melanoma patients [117]. Moreover, a growing body of evidence indicates that the antitumor efficacy of transferred T cells can be markedly improved by ablation of the host immune system before ACT. It has been reported that adoptive transfer of TILs after lymphodepleting regimen led to objective clinical responses in 51% of the patients [112]. The depletion of endogenous immune cells can be achieved via total body irradiation or chemotherapy. In the setting of ACT for the treatment of solid tumors, the lymphodepleting regimens are termed nonmyeloablative, if they transiently deplete lymphocytes and do not lethally damage the host bone marrow [118, 119] or myeloablative, if transfer of BM or hematopoietic stem cells (HSCs) is required to replace the lethally damaged host’s marrow [120]. The augmented efficacy of tumor-reactive T cells in the lymphopenic environment is due to several factors including elimination of immunosuppressive cells such as T regulatory cells (Tregs) [121-123] and depletion of endogenous cells that compete for activating cytokines [118].

Treg cells possess a $CD4^+CD25^+FoxP3^+$ phenotype and are considered the crucial mediators of peripheral tolerance [124, 125]. They arise both in the thymus and through the conversion of $FoxP3^-CD4^+$ T cells in the periphery [125, 126]. The elevated numbers of Tregs were detected in patients with late-stage tumor [125]. In addition, the suppressive effect of Tregs on function of TILs has been recently reported [127, 128], and Tregs specific for melanoma antigens have been described [129]. Also, Tregs are known to have increased capacity to consume IL-2 [130, 131], which is considered a T-cell growth factor necessary for expansion and function of tumor-specific T cell population *in vitro* and *in vivo* [112]. Thus, the removal of Tregs cells by lymphodepletion might result in enhanced antitumor reactivity of adoptively transferred $CD8^+$ T cells, not only by elimination of direct cellular inhibition but also through increased availability of IL-2. In fact, depletion of endogenous lymphocytes that compete with transferred cells for homeostatic cytokines has been shown to strongly contribute to improved efficacy of ACT [118]. The importance of the availability of

cytokines have also been demonstrated in experiments in which mice deficient for IL-7 or IL-15 showed impaired homeostatic maintenance and proliferation of memory $CD8^+$ T cells [132, 133]. Recent studies implicate that NK cells by consuming IL-15, the cytokine crucial for their survival and proliferation, serve as a one of the key effectors of the cytokine "sink" effect [134, 135]. Therefore, destruction of endogenous lymphocytes that results in an increased availability of homeostatic cytokines leads to the expansion of adoptively transferred T cell.

Another approach that may further increase ACT efficacy includes administration of T-cell growth factors that improve persistence and effectiveness of the transferred cells [112]. As delivery of early effector $CD8^+$ T cells was shown to be more effective than treatment with differentiated counterparts [115, 116, 136], exploring new strategies for *in vivo* stimulation of adoptively transferred T cells is of a great importance. Among them, cancer vaccines may become a successful method when administrated together with ATC. Thus, further optimization of the ACT treatment and relative contribution of different mechanisms of host immune manipulation to ACT therapy remains to be fully elucidated.

7. Dendritic cells as critical antigen-presenting cells in tumor immunity

One of the primary goals of cancer vaccines is to target the TAA to appropriate APCs. Dendritic cells, as the most powerful APC, play a key role in vaccine strategies. They are responsible for the initiation and regulation of T cell immunity to tumors and pathogens while at the same time preventing immune responses against self-tissues [137, 138]. This critical property of DCs is linked to environmental stimuli, which also determines their final differentiation and maturation status [137]. In the absence of inflammation, DCs remain in an immature state, and antigens that are presented to T cells without costimulation lead to T-cell anergy, deletion [139] or development of Treg cells [140]. Several stimuli, such as pathogens recognized by means of Toll-like receptors (TLR), CD40L, heat shock proteins, inflammatory cytokines, and innate lymphocytes can induce DC maturation and T cell immunity [137].

There is a substantial evidence that DCs are frequently dysfunctional in patients with advanced cancer [141]. Suppression of DC function is caused by tumor-derived factors, such as IL-10, transforming growth factor-β (TGF-β) or vascular endothelial

growth factor (VEGF). Moreover, the presence of ceramides, gangliosides or nitric oxide in tumor environment induces DC apoptosis. Dysfunctional DCs lack the ability to undergo maturation. Consequently, they do not express costimulatory molecules or cytokines which results in anegry of effector T cells and leads to the induction of regulatory Treg cells [142]. Therefore, the use of *ex vivo*-generated DCs as carriers of cancer vaccines represents an alternative approach.

A number of studies have shown that BM-derived murine DCs fused with irradiated tumor cells [143], tumor protein extracts [144] or synthetic peptide tumor epitopes [145] and matured in the presence of cytokine cocktail, induce protective immunity to subsequent tumor challenge. DCs transfected with nucleic acid encoding tumor antigens and costimulatory molecules [146] or cytokines [147] have also been effective. Furthermore, DC-based immunotherapy has been introduced to the clinic, and has proven to be feasible and nontoxic. Several reports demonstrated that DCs freshly isolated from peripheral blood or generated *ex vivo* from blood precursors and pulsed with tumor antigen were capable of eliciting specific immune responses in patients with lymphoma [148, 149], metastatic melanoma [150, 151], colon and non-small lung carcinoma [152]. However, the effectiveness of DC-based vaccines in inducing clinical responses is still below expectations [153]. Recent studies indicate that proper manipulation of *ex vivo* generated DCs is crucial for inducing effective antitumor immunity. The conditions of DC maturation have been proven to imprint their ability to secrete cytokines and different classes of chemokines as well as their immunostimulatory function and migratory activity [154]. In particular, it has been shown that DCs matured in the presence of IL-1β/TNFα/IL-6/PGE_2 cocktail expressed high levels of costimulatory molecules, however, they had reduced ability to produce IL-12. “Type-1 polarized” DCs with enhanced ability to induce long-lived, effective tumor-specific T cells are generated by maturation with type-1 and type-2 interferons together with TLR ligands [155, 156]. Importantly, several studies demonstrated that matured DCs not only activate T cell immunity but also contribute to peripheral tolerance by promoting clonal expansion of Tregs [157]. Lack or low levels of vaccine-induced Treg cells are crucial for the successful therapy [142, 158, 159]. However, while most of DC maturation protocols have largely focused on the ability of DCs to induce antigen-specific effector cells, little is known about their ability to concurrently generate Tregs. As Tregs limit primary responses to tumor/self-antigens in a tumor-bearing host and prevent generation of memory T cells [125, 126, 160], it is important

to identify stimuli that trigger a desired DC-maturation profile leading to preferential induction of tumor-specific CTLs but limited generation of Treg cells.

In summary, DCs as critical APCs that initiate and regulate immunity are critical for effective vaccination. It is well known that these cells play an important role in shaping the host response to tumors. However, the complexity of the DC biology requires rational manipulation of DCs to achieve therapeutic immunity. Therefore, further research in needed to analyze the immune response induced in patients by *ex vivo* generated DCs that are activated through different pathways. Furthermore, combining DC vaccination with other therapies might significantly increase the efficacy of antitumor treatment. For example, recent studies indicate that immune ablation may improve outcome of vaccination with DCs [161]. Although *ex vivo* generated DCs provide a unique opportunity to avoid tumor-induced DC dysfunction and allows for precise manipulation of patients' DCs, the need for specialized cell culture facilities for the *ex vivo* manipulation markedly limits this type of approach. Thus, strategies to develop cell-free vaccines targeting endogenous DCs in patients for induction of protective immune responses are now under intensive studies.

8. The role of activating/inhibitory Fcgamma receptors (FcγRs) in immunotherapy of cancer

A hallmark of immune system is its ability to maintain balance between extremes of reactivity and quiescence. The positive and negative signals, which either activate or inhibit immune responses, are tightly modulated through the action of cytokines, downstream signaling pathways and cell-cell contact. The failure to keep this balance may result in aberrant responses leading to tolerance or autoreactivity.

FcRs for IgG antibody represent a system responsible for generation of well-balanced immune response [162]. This system comprises both activating and inhibitory FcγRs which carry either immune tyrosine activation (ITAM) or inhibitory (ITIM) motif in their cytoplasmic domain [163]. To date, three different classes of activating FcRs (FcγRI (CD64), FcγRIII (CD16), FcγRIV) and one inhibitory FcγRIIb (CD32b) have been described in mice (Fig. 1). The human activating FcR system is more complex as it consists of FcγRI, two types of FcγRII, FcγRIIIA and unique FcγRIIIB

that is attached to the cell membrane by glycosylphosphotidylinositol (GPI). With the exception of human FcγRIIA and FcγRIIC, functional activating FcR is usually formed of ligand-binding α-chain and a signal-transducing γ-chain dimer. The signaling pathways initiated by the different activating receptors are similar and start with tyrosine phosphorylation of the ITAMs by SRC kinase family members [164]. This leads to the recruitment of SYK-family kinases which in turn activates a number of other signal-transduction molecules including phosphoinositide 3-kinase (PI3K) [165]. The activation of PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) that recruits phospholipase Cγ (PLCγ) to the cell membrane which results in increased intracellular calcium level and triggers further downstream signaling events [166, 167]. On the other hand, crosslinking of the single-chain inhibitory receptor FcγRIIB by immune complexes (ICs) leads to phosphorylation of the ITIM and subsequent recruitment of SRC-homology-2-domain-containing inositol-5-phosphatase (SHIP) which interferes with activating signaling pathways by hydrolysing phosphoinositide intermediates [168].

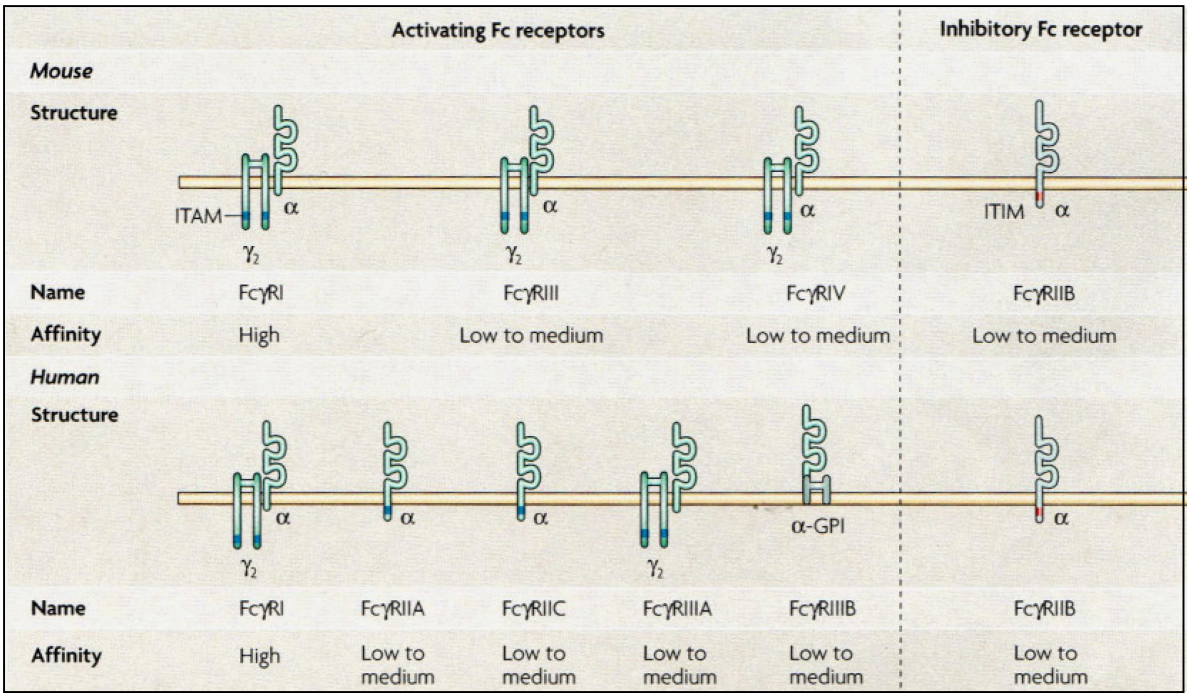


Figure 1. The family of Fc receptors for IgG [169]

Innate immune effector cells, such as mast cells, basophilis, neutrofils, monocytes and dendritic cells express both activating and inhibitory FcγR. For instance,

in mice, monocytes and macrophages express all activating and inhibitory FcγRs (FcγRI–FcγRIV), neutrophils mainly express the inhibitory FcγRIIB and the activating FcγRIII and FcγRIV, whereas the expression of FcγRI, FcγRIIB and FcγRIII dominates on DCs (reviewed in ref. [169]). There are two cell types that express only one type of the receptor: NK cells solely express the activating receptor FcγRIII whereas B cells are characterized by the expression of inhibitory receptor FcγRIIB only [169].

The balance between activating and inhibitory receptors modulates the physiologic consequences of cell-bound IgG and antigen-IgG immune complexes. Engagement of activating FcγRs mediates phagocytosis, antibody-dependent cytotoxicity and release of cytokines. In contrast, inhibitory FcγRs act as negative regulators of innate and adaptive immunity [170]. Importantly, recent studies have demonstrated that activating FcγRs expressed on DCs are also involved in antigen presentation as they mediate internalization of ICs and opsonized dying cells, such as those generated during antibody therapy of cancer [170]. Moreover, targeting of antigens to activating FcγRs on DCs by means of ICs was shown to result not only in enhanced generation of $CD4^+$ T cells but also in immunity that includes cross-presentation of TAAs to $CD8^+$ T cells. In addition, the selective blockade of inhibitory Fcγ receptors is associated with DC activation [171] and DCs from mice deficient in the inhibitory FcγRII have a more activated phenotype [172]. Altogether these studies suggest that the balance between activating and inhibitory FcγRs has a major impact on DC activation *in vivo* and, if properly manipulated, may lead to induction of desired type of immune responses. Indeed, it has recently been shown that selective targeting of activating FcγRs results in the generation of a type I interferon response program in human monocytes and monocyte-derived DCs [173]. It is known that although all classes of FcγRs are capable of binding IgG immune complexes, individual Fc receptors display significantly different affinities for IgG subclasses [174]. For example, activating Fcγ receptors possess higher affinities for murine IgG2a, IgG2b or human IgG1, IgG3 isotypes whereas inhibitory Fcγ receptor strongly binds murine IgG3. Therefore, the differences in the ratios of activating-to-inhibitory receptor binding by the presented antigenic complex may predict the ability of DCs to induce immune responses or tolerance [174]. These findings have important implications for understanding the pathophysiology of ICs diseases and for optimizing the efficacy of therapeutic antibodies. They also suggest novel strategies for targeting antigens to the

activating FcγRs on DCs to generate antigen specicific immunity in tumor-bearing hosts.

9. Oncolytic viruses: a novel form of immunotherapy

Oncolytic virotherapy represents a new form of cancer treatment that is under intensive evaluation in both preclinical and clinical settings. This strategy is based on selective infection of tumor cells (vs. normal cells) by certain naturally occurring or engineered lytic viruses, which leads to specific lysis of tumor cells. Antitumor activity of virotherapy acts through multiple mechanisms including not only oncolysis but also the induction of tumor-specific apoptosis and virus-mediated syncytium formation [175]. In addition, oncolytic viruses can be further engineered to delete immunosuppressive viral components and to insert transgenes that enhance antitumor immunity. Studies with murine tumor models confirmed the generation of cytotoxic $CD8^+$ T cells against tumor antigens expressed by the oncolytic viruses [176]. As they are relatively simply to construct and provide high levels of transgene expression, engineered viruses represent an attractive expression system.

The efficacy of recombinant viruses as vaccine vectors was initially shown using vaccinia virus [177, 178]. Recombinant vaccinia virus (rVV) and other related poxviruses have served as vectors for gene delivery and as vaccines for infectious disease and cancer for many years [179]. However, it is only in the last decade that VV has been specifically genetically engineered and explored as a recombinant oncolytic vaccinia virus (rOVV). Poxviruses are a family of large DNA genome viruses. The VV viral genome is composed of ~190 kb dsDNA encoding about 200 viral genes. The virus relies on both its own encoded proteins and host cell proteins that function for its life cycle, allowing for rapid, efficient transcription, translation and replication in the cytoplasm of the host cell. In tissue culture, VV can infect and replicate in many types of cell lines derived from mouse to man. A specific cell receptor for VV has not been identified, and the broad tropism of the virus implies that many receptors or nonspecific cell-association events may occur. VV strains may possess a natural tumor tropism as it activates cell-signaling pathways and alters the progression of the cell cycle upon infection by inactivation of the p53 tumor suppressor gene [180]. This may contribute to more efficient replication of the virus in rapidly growing cells such as many cancer cells

[181]. A number of strategies have been designed to further enhance viral replication in cancer cells and reduce replication in normal cells and thus maximize its safety. Early work by Moss and associates demonstrated that viral genes encoding both thymidine kinase (TK) and vaccinia growth factor (VGF) are virulence genes that are needed for efficient viral replication in normal cells *in vivo*. TK has a key function in the synthesis of DNA and thereby in cell division, as it is a part of the unique reaction chain to introduce deoxythymidine into DNA [182]. Mammalian cells contain two different TK isoenzymes, cytosolic Thymidine Kinase 1 (TK1) and mitochondrial Thymidine Kinase 2 (TK2). The latter form is located in mitochondria and is cell cycle-independent [183], whereas the cytosolic enzyme dominates in replicating cells but is absent in resting ones [184]. Cancer cells are characterized by a high level of TK1 expression. This is due to the genetic instabilities that are commonly found in tumor cells such as loss of the tumor suppressor Retinoblastoma (Rb) gene [185] or an increase in cyclin activity [186, 187]. These alterations lead to the activation of transcriptional factor E2F that drives TK expression in tumor cells [188]. Therefore, TK^- mutant of the virus retains full capacity to replicate in cancer cells while its replication in nondividing cells is reduced [180].

The VGF shares sequence homology and functional properties with mammalian epidermal growth factor (EGF) [189] and is capable of binding and inducing tyrosine phosphorylation of the EGF receptor (EGFR; ErbB1) [190]. This initiates a signaling cascade that culminates in activation of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway and results in cellular proliferation [190]. Importantly, VGF may prime surrounding cells for vaccinia infection by binding to their EGFR and causing proliferation and increases in cellular TK levels [191]. Based on these findings, attempts have been made to decrease pathogenicity of wild-type virus by deletion of VGF gene in addition to the TK gene. The absence of the VGF gene prevents activation of EGFR signaling in noncancer cells and restricts the viral replication to cells with EGF pathways already activated, such as cancers cells [192]. The resulting recombinant VV with both viral genes for VGF and TK deleted retains its ability to target and replicate in cancer cells both *in vitro* and *in vivo*, and displays significant oncolytic potency in multiple tumor models [180]. Therefore, the selective replication of rOVV in tumor leads to significant decreases in *in vivo* toxicity of the virus as confirmed in animal models and clinical trails. Importantly, the systemic efficacy that is required for effective treatment of metastatic cancer has

been reported after intratumoral injection of rOVV expressing GM-CSF in patients with melanoma, non-small cell lung and liver tumors [193].

Another important feature of vaccinia-virus-based vaccines is their ability to break $CD4^+CD25^+$ Treg-mediated CD8 tolerance through TLR-dependent and -independent pathways [194, 195]. Several lines of evidence indicate that rVV elicits innate immune responses through the TLR2/MyD88-dependent pathway, resulting in the production of proinflammatory cytokines, and a TLR-independent pathway, leading to the activation of IFN-γ *in vitro* and *in vivo* [195]. Because sustained stimulation of TLRs of the innate immunity is required for breaking established Treg-mediated tolerance *in vivo* and the virus can provide TLR signals, this unique potency of rVV or rOVV as a vaccine vehicle can lead to activation of host defense and protect the mice from tumor challenge [194, 196].

Altogether, these findings suggest that virotherapy using rOVV could become a new paradigm for safe and practical therapeutic vaccines for cancer. It represents an attractive, novel approach with potential advantages over the conventional cancer vaccines including diverse, personalized native TAAs released from oncolysis and local inflammation that augments adaptive immune responses. With the flexibility in further engineering and combination with antitumor drugs and/or with cell therapies, oncolytic virotherapy could become a potent treatment that may be superior to other cancer vaccine strategies.

V. PROJECT AIMS

A major challenge for inducing antitumor immune responses with therapeutic cancer vaccines in tumor-bearing hosts relates to achieving efficient uptake and processing of tumor antigens by dendritic cells to preferentially activate T effector cells rather than Treg cells. The overall aim of my experimental studies was to explore immunization strategies for therapeutic vaccines expressing a CD166 cross-reactive mimotope of GD2 ganglioside, 47-LDA, to provide protection against primary and metastatic tumors in NXS2-neuroblastoma-bearing syngeneic mice. It was hypothesized that preferential delivery of the 47-LDA polypeptide to activating FcγRs expressed on DCs would enhance the adjuvant potency of the mimetic vaccine by generation of high numbers of T effector cells and reduced expansion of Treg cells. To achieve this goal the following experiments were performed:

1. The 47-LDA polypeptide was expressed in the context of the murine IgG2a Fc portion and analyzed for its interaction with BM-derived DCs.
2. The induction of tumor-specific Th1 and CTL responses as well as Treg cells were investigated after immunization of A/J mice with 47-LDA-Fcγ2a- and 47-LDA polypeptide-coated DC vaccines.
3. The therapeutic efficacy of DC vaccine expressing 47-LDA-Fcγ2a fusion protein or 47-LDA polypeptide was evaluated during ACT of antigen-experienced $CD8^+$ T cells in syngeneic NXS2 primary tumor-bearing mice. Prior to the ACT and DC vaccination, the tumor-bearing mice were lymphodepleted by nonmyeloablative TBI or a myeloablative regimen that required BM transplantation to facilitate expansion of tumor-specific T cells.
4. The 47-LDA-Fcγ2a- and 47-LDA-DC vaccines were tested for their ability to induce antitumor immune responses capable of inhibiting metastatic disease after adoptive transfer of antigen-experienced $CD8^+$ T cells or naïve splenocytes to lymphodepleted mice that had the primary tumor resected prior to the treatment initiation. The tumor-free mice and mice with visible metastasis were analyzed for the presence of cellular responses.
5. To determine that the antitumor effect of immunization with the fusion protein was not limited to the DC vaccine and ACT in an irradiated tumor-

bearing host, 47-LDA-Fcγ2a construct was expressed in the oncolytic TK^-, VGF^- mutant of recombinant vaccinia virus and examined for its therapeutic efficacy in the syngeneic NXS2 tumor-bearing mice. The NXS2-challenged mice which remained tumor free were further analyzed for the presence of tumor-specific immune memory.

VI. MATERIALS AND METHODS

1. Animals and cell lines

Female A/J mice, 6-8 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, ME). The animals were housed under pathogen-free conditions in the Animal Facility at Roswell Park Cancer Institute (RPCI), Buffalo, NY and the experimental procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the RPCI.

The murine NXS2 neuroblastoma cell line (provided by Dr. R. A. Reisfeld, The Scripps Res. Inst. La Jolla, CA) is a hybrid between the GD2-negative C1300 murine neuroblastoma (A/J background) and GD2-positive murine dorsal root ganglioma cells. The hybrid cell line was shown to be MHC class I syngeneic to A/J mice by its H-2K^k-positive/H-2K^b-negative phenotype [197]. NXS2-*RLuc* cells were obtained by stable transfection with pcDNA 3.1 vector expressing Renilla Luciferase (RLuc) using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) followed by selection in hygromycin-containing medium. The 14G2a hybridoma cell line secreting GD2-specific mAb [198] was provided by Dr. R. A. Reisfeld (The Scripps Res. Inst.). Human 293T fibroblasts, monkey kidney fibroblasts (CV1), human cervical adenocarcinoma (HeLa S3) and human TK^- cells were obtained from American Type Culture Collection (ATCC), Rockville, MD. The cells were cultured at 37^0C in a 5% CO_2 in DMEM medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Life Technologies) and gentamicin (Sigma Co., St. Louis, MO).

2. Generation of 47-LDA-Fcγ2a fusion protein

The construction of 47-LDA expression vector, consisting of tissue plasminogen activator secretory (tPA) signal sequence, two universal Th peptides PADRE (AKFVAAWTLKAAA; ref. [199]) and V3 loop of the HIV gp120 glycoprotein (CKRKIHIGPGQAFYT; ref. [200]) together with the 47-LDA mimetic peptide was reported elsewhere [57]. The murine 47-LDA-Fcγ2a fusion protein was generated by inserting the 47-LDA polypeptide coding sequence between the hEF1-HTLV promoter

and the mouse IgG2a Fc region of the pFUSE-mIgG2Aa-Fc1 vector (InvivoGen, San Diego, CA) using the *EcoR*I and *Bgl*II restriction enzyme cleavage sites. Stable transfection of 293T cells was done with the 47-LDA-Fcγ2a fusion protein construct or sham vector using Lipofectamine Reagent followed by selection in zeocin-containing medium. The 47-LDA-Fcγ2a fusion protein was purified from culture supernatants of the transfected cells on Protein G column (GE Healthcare, Bio-Sciences Corp, Piscataway, NJ). The fusion protein was analyzed by SDS-15% PAGE and immunoblotting with 14G2a mAb followed by ECL plus Western blotting detection system (Amersham Pharmacia Biotech) according to the protocol of the manufacturer.

3. Mice immunization

DCs were generated *in vitro* from bone marrow precursors as previously described [201]. Briefly, BM cells were harvested from the tibias and femurs of 6- to 8-week-old female A/J mice and then cultured in complete medium supplemented with 10 ng/ml GM-CSF at 37^{o}C for 6 days. The medium was replenished every 2-3 days. On day 7, most of the nonadherent cells had acquired DC morphology and were CD11c high and CD80, CD86, CD40, and MHC class II low, as determined by flow cytometric analyses. DCs were pulsed for 5 h with 10 μg/ml of 47-LDA-Fcγ2a fusion protein or 47-LDA polypeptide (New England Peptide, LLC, Gardner, MA), incubated with 1.0 μg/ml LPS (Sigma) for 1 h to induce maturation, washed and injected i.v. to mice (2 x 10^6 cells per mouse). 20 μg of DNA plasmid-encoded IL-15 and IL-21 were injected i.m. at the time of vaccination and five days later, respectively [61]. Mice were immunized every two weeks for the total of three immunizations.

4. Flow cytometry

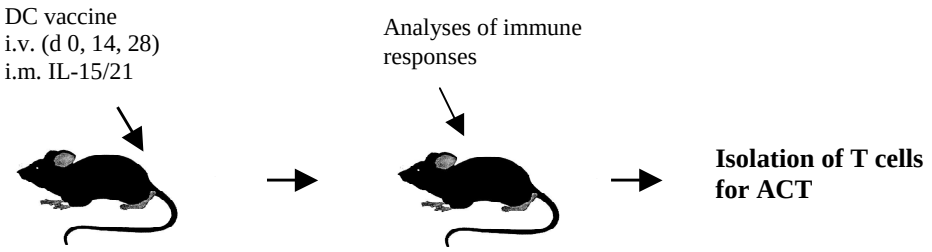
Immature DCs were incubated with biotin-labeled 47-LDA-Fcγ2a fusion protein or 47-LDA polypeptide followed by streptavidin-conjugated PE (Phycoerythrin). For some experiments, DCs were stained with the following mAbs: anti-CD11c-FITC, anti-CD80-PE, anti-CD86-PE, anti-CD40-PE, or anti-MHC class II-PE (BD Bioscience, San Jose, CA). Splenocytes were labeled with anti-CD4-PE, anti-CD8-PE, anti-CD25-FITC

(BD Bioscience) or the relevant isotype controls. Prior to specific antibody staining, cells were incubated with Fc blocker (anti-CD16/CD32 mAb) for 10 min. The cells were washed twice in HBSS containing 0.01% sodium azide and 1% FCS, fixed with 1% paraformaldehyde, and stored at 4°C in the dark before analyses. Background staining was assessed using isotype controls which included the appropriate fluorochrome-conjugated or unconjugated mouse IgG1, IgG2a or IgG2b (BD Bioscience). The numbers of Treg cells in axillary, brachial lymph nodes and spleen were determined by intracellular staining of $CD4^+CD25^+$ lymphocytes with anti-FoxP3-AlexaFluor 647 mAb (eBioscience, San Diego, CA) according to the manufacturer's protocol. The number of $CD4^+$ and $CD8^+$ splenocytes secreting IFN-γ or TNF-α was determined using anti-CD4-FITC and anti-CD8-FITC mAbs in conjunction with anti-IFN-γ-PE or anti-TNF-α-PE mAb (BD Bioscience). The intracellular expression of IL-12p70 in LPS-stimulated DCs was measured by staining with rat anti-mouse IL-12p70 mAb that detects an epitope within the IL-12p35 subunit and does not cross-react with IL-12p40 (R&D Systems, Inc.). The intracellular expression of CCL22 in LPS-stimulated DCs was determined by staining with rat anti-mouse CCL22 mAb (R&D). Sorting of 47-LDA-Fcγ2a$^+$, 47-LDA polytope$^+$ or $CD86^+$ DC was performed on BD FACSAria™ Flow Cytometer (BD, Franklin Lakes, NJ). All flow cytometric evaluations were performed on FACScan or FACSCalibur flow cytometer. After gating on forward and side scatter parameters, at least 10,000-gated events were routinely acquired and analyzed using CellQuest software (BD Biosciences).

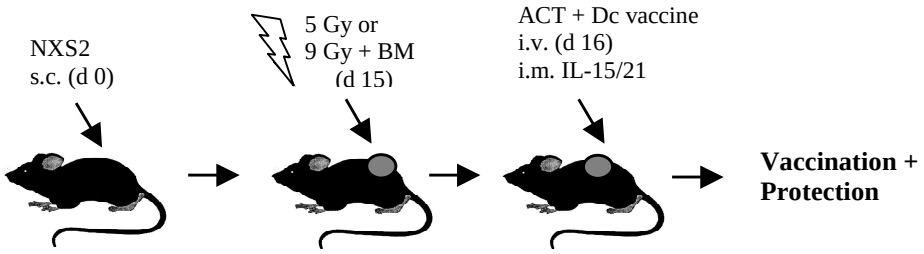
5. Adoptive transfer of T cells

A/J mice (n = 8 - 10 per group) were injected s.c. with 2 x 10^6 NXS2 neuroblastoma cells and treated 15 days later by i.v. injection with $CD8^+$-enriched splenocytes isolated from 47-LDA-Fcγ2a- or 47-LDA polytope-DC-immunized mice, as depicted in Fig. 2A and B. The $CD8^+$ splenocytes were negatively selected using paramagnetic Microbeads conjugated to anti-mouse CD4 (L3T4) and anti-mouse CD45R (B220) mAbs (MACS; Miltenyi Biotec) according to the manufacturer's instructions. The isolated $CD8^+$ splenocytes (2 x 10^7) were incubated with LPS-matured 47-LDA polypeptide- or 47-LDA-Fc fusion protein-coated DCs. The mixtures of T cells and DCs (20:1 ratio) were

A. Induction of antitumor immune responses with 47-LDA- or 47-LDA-Fcγ2a-DCs



B. Inhibition of NXS2 tumor growth by ACT and DC vaccines



C. Inhibition of metastatic disease by ACT and DC vaccines

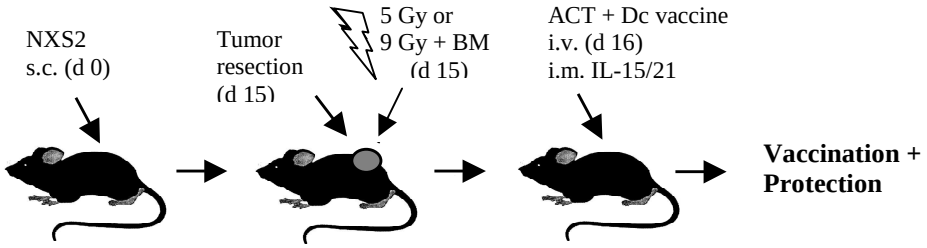


Figure 2. Schematic representation of vaccination with 47-LDA- or 47-LDA-Fcγ2a-pulsed DCs and ACT in tumor-bearing mice. (A) Vaccination with 47-LDA- or 47-LDA-Fcγ2a-pulsed DCs. A/J mice (n = 5) were immunized in a 2-week interval with 47-LDA- or 47-LDA-Fcγ2a-pulsed DCs for a total of three immunizations. Three weeks after the last immunization $CD8^+$ splenocytes were obtained by negative selection and used for the ACT in NXS2-bearing mice. (B) Inhibition of primary tumor growth by ACT and DC vaccines. A/J mice were challenged with 2 x 10^6 NXS2 cells. Fifteen days later, lymphopenia in the tumor-bearing mice was induced by 5 Gy TBI or 9 Gy TBI plus BM transfer (10^7 cells) one day prior to the adoptive transfer of antigen-experienced $CD8^+$ T cells (2 x 10^7) and DC vaccination. (C) Inhibition of metastatic disease by ACT and DC vaccine. NXS2-bearing mice underwent nonmyeloablative or myeloablative TBI at the time of tumor excision. One day later, the mice received ACT of CD8+ T cells (2 x 10^7) from the immunized mice or unseparated splenocytes from naïve mice together with the DC vaccine. Survival was defined as the point at which mice were sacrificed due to extensive tumor growth or development of metastases.

injected i.v. to the lymphodepleted NXS2 tumor-bearing mice in the presence of rmIL-2 (1 μg/dose) as described [202]. Lymphopenia in the tumor-bearing mice was induced by 5 Gy nonmyeloablative TBI or 9 Gy myeloablative TBI plus BM transfer (10^7 cells) one day prior to the ACT and vaccination (Fig. 2B). Control mice bearing established s.c. NXS2 tumors were irradiated with 5 Gy or 9 Gy plus BM transplantation. Mice were immunized in a 2-week interval with the DC vaccine in the presence of IL-15 and IL-21 vectors as described [61]. Tumor growth was monitored by measuring s.c. tumors once to thrice a week with a microcaliper and determining tumor volume (width x length x width/2 = mm^3). Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method.

6. Immunotherapy of disseminated disease

For the immunotherapy of disseminated disease, NXS2-*Rluc*-bearing mice underwent nonmyeloablative (5 Gy) or myeloablative (9 Gy) TBI at the time of tumor resection (Fig. 2C). One day after tumor excision, the mice received ACT of splenocytes (2×10^7) from the immunized or naïve mice together with the 47-LDA-Fcγ2a-DC or 47-LDA-DC vaccine. Mice were immunized in a 2-week interval with the DC vaccine in the presence of IL-15 and IL-21 vectors as described [61]. The control mice had the primary tumor excised with or without TBI and BM transplantation. Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method.

7. *In vitro* analyses of vaccine-induced IFN-γ and TNF-α expression and T cell proliferation

Splenocytes from A/J mice immunized with the 47-LDA-Fcγ2a- or 47-LDA polypeptide-coated DCs were analyzed for IFN-γ and TNF-α expression after overnight stimulation with 47-LDA-expressing DCs at the 20:1 ratio. Cells isolated from sham vector-immunized mice served as controls. To investigate the induction of Treg cells in mice immunized with 47-LDA-DC and 47-LDA-Fcγ2a-DC vaccines, the spleen, axillary and brachial lymph nodes

were removed from the immunized mice three weeks after the last immunization analyzed for numbers of Treg cells by staining with anti-CD4-PE, anti-CD25-FITC and anti-FoxP3-AlexaFluor 647 mAb, or the relevant isotype controls. To analyze the effect of Treg cells on Teff cell proliferation, $CD8^+$ T cells from the 47-LDA-DC or 47-LDA-Fcγ2a-DC vaccine immunized mice were loaded with 25 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37°C and cultured with 47-LDA-expressing syngeneic DCs (ratio 20:1) for 72 h in the presence or absence of Treg cells (ratio 1:1). Cells were stained with PE-conjugated anti-CD8 mAbs and analyzed by flow cytometry. The Treg cell populations were isolated using $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi) according to the manufacturer's protocol.

To analyze proliferative $CD4^+$ and $CD8^+$ T cell responses in tumor-bearing and tumor-free mice after ACT and DC vaccination, splenocytes were labeled with 25 µM CFSE and incubated with 47-LDA-expressing DCs for 72 h. Cells were stained with PE-conjugated anti-CD4 or anti-CD8 mAbs and analyzed by flow cytometry.

8. CTL assay

Splenocytes were cultured with 47-LDA-expressing DC at the 20:1 ratio in 15% T cell stimulatory factor (T-STIM™ Culture Supplement, Collaborative Biomedical Products, Bedford, MA) as a source of exogenous IL-2. After three days of stimulation, cells were split and cultured in medium supplemented with murine rIL-2 (0.3 ng/ml) (BD Bioscience). Prior to stimulation, $CD8^+$ T cells were isolated by negative selection using T cell enrichment columns (Miltenyi) according to the manufacturer's protocol. The cytolytic activity of CTLs against NXS2 tumor cells was analyzed 5 days later by a standard 4-h ^{51}Cr-release assay. The percent of specific lysis was calculated as: ([cpm experimental release - cpm spontaneous release]/[cpm maximum release - cpm spontaneous release]) x 100. Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated with medium only.

9. *In vivo* bioluminescence imaging

In vivo bioluminescence imaging was conducted on a cryogenically cooled IVIS 50 system (Xenogen Corp., Alameda, CA) coupled to a data-acquisition PC running LivingImage™ software (Xenogen Corp.). Mice were injected i.v. with coelenterazine (Prolume Ltd; 15 µg per animal) for NXS2-*RLuc*-positive tumor cell imaging. Coelenterazine was used as the initial substrate for imaging of RLuc expression. An integration time of 1 minute was used for luminescent image acquisition. The mean number of photons per square centimeter per second per steradian in the region of interest (ROI) was determined.

10. Generation of oncolytic vaccinia viruses expressing green fluorescence protein (EGFP) and the 47-LDA-Fcγ2a fusion protein

The rOVV expressing enhanced green fluorescence protein (EGFP) and 47-LDA-Fcγ2a fusion protein have been generated by homologous recombination in CV-1 cells [203] using the VSC20 vaccinia virus and the vaccinia shuttle plasmid pSEL-EGFP and pCB023-47-LDA-Fcγ2a, respectively. The parental vaccinia virus VSC20 with lacZ gene cloned in place of VGF gene was obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD). The pCB023 and pSEL-EGFP vaccinia shuttle plasmids were obtained from Dr. D. Bartlett (University of Pittsburgh Cancer Institute, Pittsburgh, PA). The pCB023-47-LDA-Fcγ2a shuttle plasmid was generated by cloning the 47-LDA-Fcγ2a fusion protein gene into the *EcoRI* and *SmaI* restriction enzyme sites of pCB023. After the DNA sequence verification, the secretion of the 47-LDA-Fcγ2a fusion protein into culture supernatant of 293T cell transfectants was confirmed by immunoblotting with 14G2a mAb. Confluent wells of CV1 cells were infected for 2 h at 37°C with 0.05 plaques forming units (PFUs) of VSC20 per cell in 1.0 ml of DMEM-2% FBS. Supernatants were removed, and a liposomal transfection of pSEL-EGFP or pCB023-47-LDA-Fcγ2a was performed using 0.5 ml of DMEM-2% FBS containing 4 µg of plasmid DNA and 10 µl of liposomes/well at 37°C for 4 h. The cells were overlaid with 3.0 ml of DMEM-10% FBS. After 2 days of incubation, cells were trypsinized, sonicated and freeze-thaw for a total of three cycles. Serial dilutions of cell lysate were used to infect TK^- cells. After 24 h, the viral inoculum was aspirated and

cells were overlaid with 3 ml of low melting agarose (BioProducts, Rockland, ME) containing bromodeoxyuridine (BrdU, final concentration 25 μg/ml; Sigma), 0.5 % gentamicin (Sigma) and 1/120 vol of 4 % l5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma). The well-separated plaques of rOVV-EGFP and rOVV-47-LDA-Fcγ2a viruses were isolated, resuspended in DMEM-2% FBS, and used to reinfect further TK^- cells. After five cycles of selection, the positive plaques were amplificated on HeLa cells, purified over the sucrose gradient and tittered on TK^- cells and used for *in vitro* and *in vivo* studies in NXS2 cells. The expression of EGFP in rOVV-EGFP-infected NXS2 cells was confirmed by immunofluorescence microscopy, whereas secretion of the 47-LDA-Fcγ2a fusion protein from rOVV-47-LDA-Fcγ2a-infected NXS2 cells was determined by immunoblotting with 14G2a mAb.

11. Titration of vaccinia virus by plaque assay

Serial dilutions of the virus stock were prepared and used to infect the confluent TK^- cells (0.5 ml of virus dilution/well). After 2 h of incubation (37^0C, 5% CO_2), the cells were overlaid with 2 ml of complete DMEM medium supplemented with 2% FBS and kept for 2 days in CO_2 incubator at 37^0C. The viral plaques were visualized by removing the medium and adding 0.5 ml of 0.1 % crystal violet (Sigma) in 20% ethanol for 5 min. The plaques appeared as 1- to 2-mm-diameter areas of diminished staining due to retraction, rounding and detachment of infected cells.

12. Viral biodistribution

NXS2-tumor bearing A/J mice were injected i.v. or intratumoral with 10^8 pfu of rOVV-EGFP. In some experiments, adoptive transfer of NXS2-specific $CD8^+$ T cells (10^7 cells) loaded with rOVV-EGFP was used to chaperone the virus and deliver it to the tumor site. At day 4th following viral administration, samples of normal tissues and tumor were harvested, washed in PBS and stained with X-Gal in an iron solution of 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$ at 37°C for 15 minutes. The rOVV-infected tissue generated an indigo-blue color as a result of β-galactosidase expression. To determine the level of viruses in all samples, organs and tumor were

homogenized, freezed and thawed for a total of 3 cycles and titered on TK^- cells by standard plaque assay.

13. Virotherapy of NXS2-tumor bearing A/J mice

For the oncolytic virotherapy-based vaccine, A/J mice (n = 10) were injected s.c. with 2 x 10^6 NXS2 cells and treated 15 days later with i.v. injection of 10^8 pfu of the rOVV-47-LDA-Fcγ2a or rOVV-EGFP vector. Tumor-bearing mice that were treated with PBS served as controls. Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method.

14. Statistical analyses

The statistical significance of the difference between groups was performed using a two-tailed Student's t test assuming equal variance. The P values for the pairwise group comparisons for the average tumor growth were computed using the nonparametric Wilcoxon's rank-sum test. Kaplan-Meier survival plots were prepared and median survival times were determined for NXS2-challenged groups of mice. Statistical differences in the survival across groups were assessed using the logrank Mantel-Cox method. Data were presented as arithmetic mean ± SD and analyzed using the JMP program (SAS Institute Inc., Cary, NC) on a Windows-based platform.

VII. RESULTS

1. Generation and characterization of murine 47-LDA-Fcγ2a fusion protein

The murine 47-LDA-Fcγ2a fusion protein was generated by inserting the relevant coding sequence (including the tissue plasminogen activator secretory (tPA) signal sequence, two universal Th peptides, and the 47-LDA mimotope) in-frame between the hEF1-HTLV promoter and the mouse IgG2a Fc region of the pFUSE-mIgG2Aa-Fc1 vector. After sequencing of the 47-LDA-Fcγ2a construct to confirm the presence of an uncorrupted open reading frame, the immunofluorescence staining of 47-LDA-Fcγ2-transfected 293T cells with 14G2a mAb followed by flow cytometry analysis was done 48 h after transfection to determine expression of the fusion protein (Fig. 3A). The secretion of the fusion protein from 47-LDA-Fcγ2a stable transfectants, selected in zeocin-containing medium, was confirmed by immunoblotting of the culture supernatant with 14G2a mAb. As shown in Fig. 3C, a prominent band of 31.9 kDa was detected with 14G2a mAb in the culture supernatant harvested from 47-LDA-Fcγ2a transfectants. No band was present in the supernatant prepared from cell transfected with the sham plasmid. The 47-LDA polypeptide of molecular mass of 6.6 kDa, which was included in the analysis as a positive control, was clearly recognized by 14G2a mAb. Altogether, these results showed that the GD2 mimotope expressed in the context of 47-LDA-Fcγ2a fusion protein retained its native antigenic determinants of the synthetic peptide recognized by 14G2a mAb.

The binding of the recombinant 47-LDA-Fcγ2a fusion protein to DCs was analyzed by flow cytometry analysis. DCs were generated *in vitro* from bone marrow precursors as described in Material and Methods section and incubated with biotin-labeled 47-LDA-Fcγ2a fusion protein or 47-LDA polypeptide followed by streptavidin-PE and FITC-conjugated CD11c-specific mAb to confirm their DC phenotype. Fig. 4 shows that over 50% of $CD11c^+$ cells were positively stained for the 47-LDA-Fcγ2a fusion protein or for the biotin-labeled 47-LDA polypeptide. The binding of 47-LDA-Fcγ2a fusion protein, but not 47-LDA polypeptide, was inhibited by incubating DCs with the FcγR blocking antibody (CD16/32) indicating a specific interaction between the 47-LDA-Fcγ2a fusion protein and its cellular ligands. It is noteworthy however that the binding of 47-LDA-Fcγ2a fusion protein to the FcγRs had no effect on DC

maturation since addition of LPS was necessary to upregulate the surface expression of costimulatory molecules MHC class II, CD86, CD40, and CD86 (data not shown).

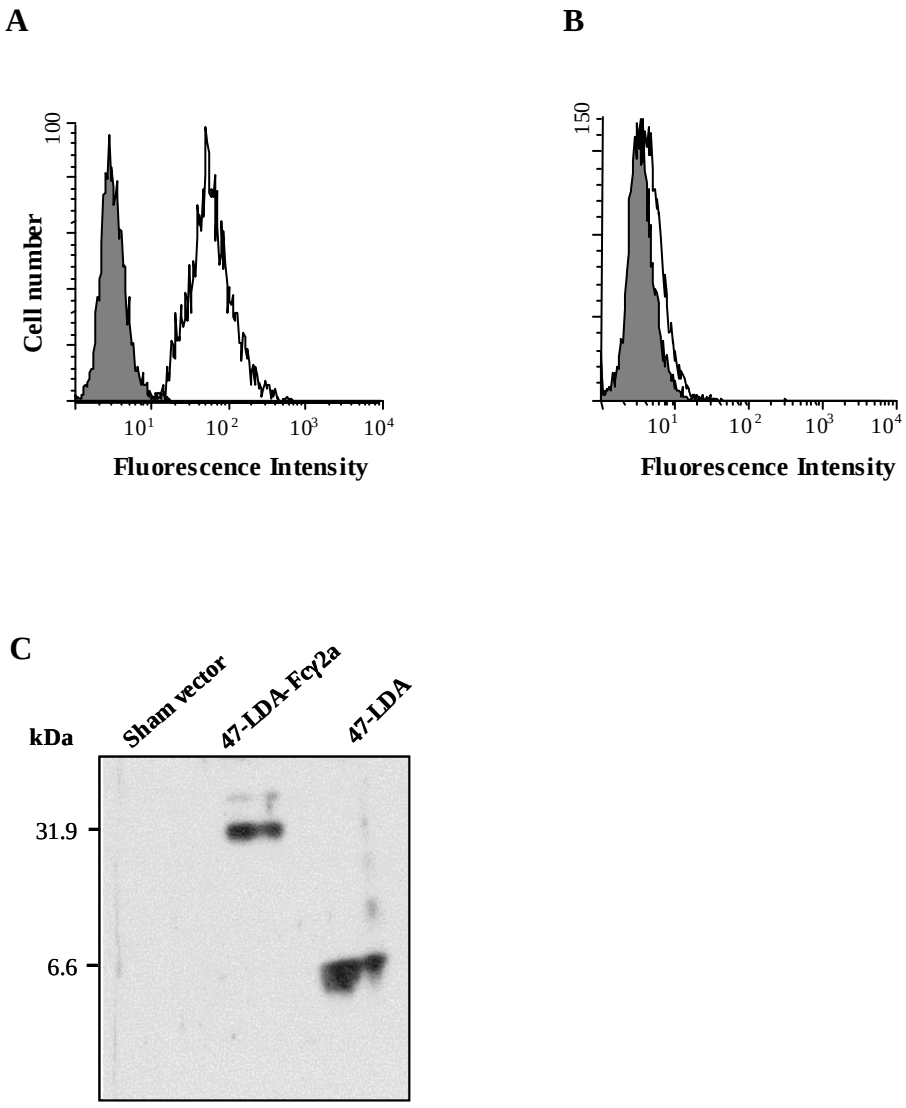


Figure 3. Flow cytometry and immunoblotting analyses of 47-LDA-Fcγ2a expression in 293T transfectants. 293T fibroblasts were transfected with 47-LDA-Fcγ2a fusion protein construct (A) or sham vector (B). The transgene expression was analyzed 48 h after transfection by intracellular staining with 14G2a mAb followed by streptavidin-PE. The light gray area denotes transfectants stained with the streptavidin-PE only. (C) Western blotting of 47-LDA-Fcγ2a fusion protein and 47-LDA polypeptide with biotinylated 14G2a mAb and streptavidin-HRP.

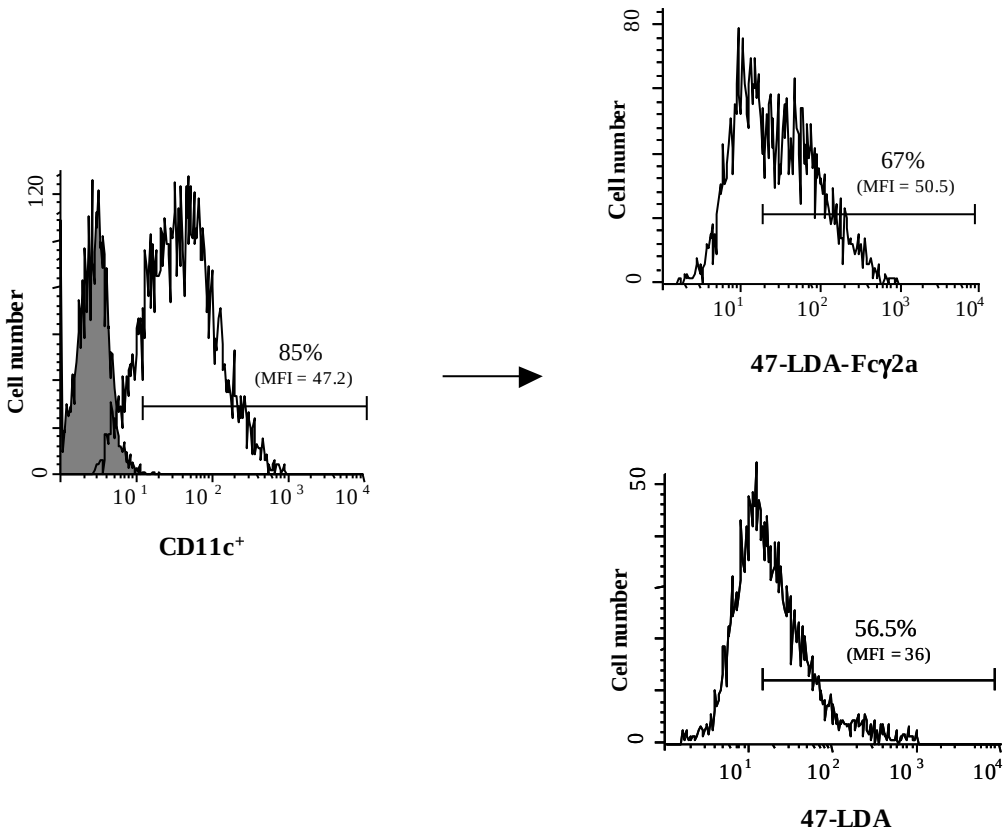


Figure 4. Binding of 47-LDA-Fcγ2a fusion protein and 47-LDA polypeptide to immature $CD11c^+$ DCs. BM-derived DCs were stained with FITC-conjugated CD11c-specific mAb in combination with biotinylated 47-LDA-Fcγ2a or 47-LDA polypeptide followed by streptavidin-PE and analyzed by flow cytometry. Filled histogram corresponds to isotype control. The percent and mean fluorescent intensity (MFI) of 47-LDA-Fcγ2a and 47-LDA on CD11c-positive DCs are given. Arrow indicates the source of cells in the histograms. Data are from one representative experiment of three performed.

2. Differential expression of IL-12 and CCL22 in 47-LDA-Fcγ2a$^+$ and 47-LDA$^+$ DCs

Because the activating and inhibitory FcγRs are critical for the modulation of effector immune responses during administration of antigens to DCs in the form of immune complexes [172, 204] and IL-12 is a key cytokine involved in the generation of type-1 immunity [205], it was next investigated whether DCs that interact with 47-LDA-Fcγ2a fusion protein or 47-LDA polypeptide differ in their ability to express IL-12p70 upon stimulation with LPS. For these experiments, 47-LDA-Fcγ2a$^+$ and 47-LDA$^+$ DCs as well as their negative counterparts were obtained by cell sorting. The sorted DC populations were stimulated overnight with 1 μg/ml of LPS and analyzed for IL-12p70 expression by intracellular staining and a flow cytometric analysis. Figure 5A shows that 47-LDA-Fcγ2a$^+$ DCs expressed significantly higher levels of IL-12p70 than their negative counterparts after stimulation with LPS (P = 0.008), whereas comparable levels of IL-12p70 were measured in 47-LDA$^+$ and 47-LDA$^-$ DC populations (Fig 5B). A parallel expression study of the Treg-attracting CCL22 chemokine [154, 206] in the sorted and LPS-stimulated 47-LDA-Fcγ2a$^+$ DCs and 47-LDA$^+$ revealed approximately twofold higher numbers of CCL22-positive cells in the latter DC population (Fig. 5C and D; P = 0.037).

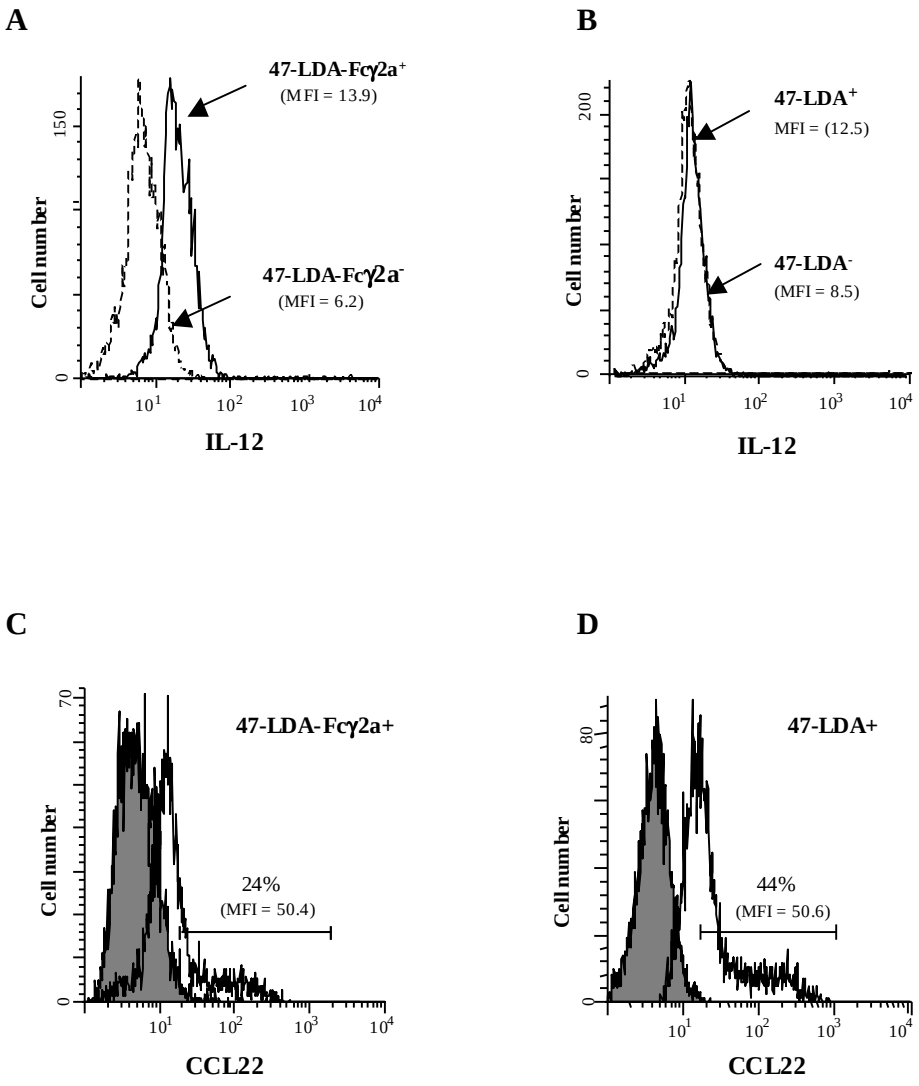


Figure 5. Flow cytometry analysis of IL-12p70 and CCL22 in 47-LDA-Fcγ2a$^+$ and 47-LDA$^+$ DCs. Intracellular expression of IL-12p70 by 47-LDA-Fcγ2a$^+$, 47-LDA$^+$ DCs and their negative counterparts after LPS stimulation (A and B). Immature DCs were stained with biotinylated 47-LDA-Fcγ2a fusion protein or 47-LDA polypeptide followed by streptavidin-PE. The positive and negative populations were sorted on BD FACSAria^{TM} flow cytometer, incubated overnight with 1 μg/ml LPS and analyzed for IL-12p70 expression by intracellular staining with rat anti-mouse IL-12p70 mAb, specific for the IL-12p35 subunit, followed by goat anti-rat secondary antibody. Background staining (MFI < 2.6) was assessed using an isotype control Ab. (C and D) Differential expression of CCL22 in 47-LDA-Fcγ2a$^+$ and 47-LDA$^+$ DCs after LPS stimulation. The sorted 47-LDA-Fcγ2a$^+$ (C) and 47-LDA$^+$ (D) DCs were incubated 24 h with 1 μg/ml LPS and analyzed for CCL22 expression by intracellular staining with rat anti-mouse CCL22 mAb, followed by goat anti-rat secondary antibody. The MFI and percent of cells positive for CCL22 expression are indicated. Light gray area denotes background staining assessed using an isotype control Ab. Data are from one representative experiment of three performed.

3. Enhanced immunostimulatory activity of 47-LDA-Fcγ2a-pulsed DCs *in vivo*

The differences in the expression levels of IL-12 and CCL22 between LPS-stimulated 47-LDA-Fcγ2a$^+$ DC and 47-LDA$^+$ DCs suggest that these cells may have different ability to interact with Teff and Treg cells *in vivo*. Therefore, the induction of tumor-specific Th1 and CTL responses after immunization of A/J mice with 47-LDA-Fcγ2a- and 47-LDA-DC vaccines was investigated in the next set of experiments. Each DC vaccine was delivered by i.v. injection three times in a 2-week interval together with DNA plasmid-encoded IL-15 and IL-21 delivered i.m. at the time of vaccination and five days later, respectively [61]. Three weeks after the last immunization, CD4$^+$ splenocytes from the immunized mice were analyzed for IFN-γ and TNF-α production by intracellular staining and flow cytometry after overnight stimulation with 47-LDA-expressing DCs. Cells isolated from mice immunized with DCs and IL-15 and IL-21 cytokine-expressing vectors were included as controls. As shown in Fig. 6A, immunization of mice with the 47-LDA-Fcγ2a fusion protein-coated DC induced over four-fold higher numbers of IFN-γ- and TNF-α-producing CD4$^+$ cells than vaccination with the control DCs, and over two-fold higher responses than 47-LDA-coated DCs ($P = 0.002$ and $P = 0.003$, respectively). The increases in IFN-γ-producing CD4$^+$ T cell responses after 47-LDA-Fcγ2a fusion protein vaccine were associated with consistently higher CD8$^+$ T cell-mediated cytotoxic actitivites against NXS2 neuroblastoma tumor compared with those induced by the 47-LDA polytope-coated DC over a broad range of E : T ratio (Fig. 6B).

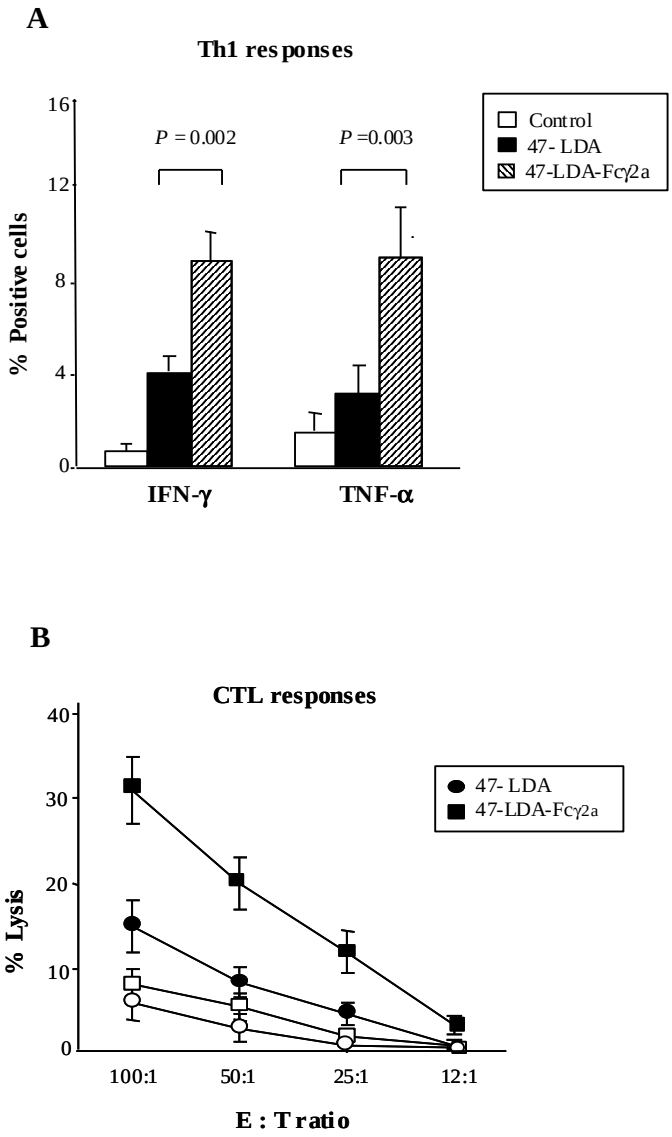


Figure 6. Induction of cellular responses by 47-LDA- and 47-LDA-Fcγ2a-DC vaccines. (A) Expression of IFN-γ and TNF-α in splenocytes of mice immunized with 47-LDA-DC and 47-LDA-Fcγ2a-DC vaccines (black and hatched bars, respectively). A/J mice (n = 5) were immunized with DCs coated with 47-LDA polypeptide or 47-LDA-Fcγ2a fusion protein after LPS-induced maturation. Cells isolated from mice immunized with LPS-treated DC served as controls (open bars). Three weeks after the last immunization, the expression of IFN-γ and TNF-α in $CD4^+$ splenocytes was analyzed by intracellular staining after overnight stimulation with DCs expressing the 47-LDA mimotope. (B) NXS2 neuroblastoma-specific CTL responses. $CD8^+$ splenocytes from mice immunized with 47-LDA-DC (●) and 47-LDA-Fcγ2a-DC (■) vaccines were obtained by negative selection. Cells were cultured with 47-LDA-expressing DCs (black symbols) or sham plasmid-transfected DCs (open symbols) at the 20:1 ratio as described in the *Materials and Methods* section. The CTL activities against NXS2 cells were analyzed in a standard ^{51}Cr-release assay. All determinations were made in triplicate samples, and the SD was <10%. Results are presented as the means ± SD of four independent experiments.

4. Immunization with 47-LDA-Fcγ2a -DC vaccine leads to generation of lower numbers of Tregs than vaccination with 47-LDA-pulsed DCs

The induction of Treg cells after 47-LDA-Fcγ2a- and 47-LDA-DC vaccines was examined by a flow cytometric analysis of $CD4^+CD25^+FoxP3^+$ lymphocytes in the axillary, brachial lymph nodes and spleen. In the axillary lymph nodes of 47-LDA-Fcγ2a-immunized mice, the absolute numbers of $FoxP3^+$ cells within the $CD4^+CD25^+$ population ranged from 0.17 x 10^6 to 0.24 x 10^6 cells (Fig. 7A) and were ~2-fold lower compared to the animals immunized with the 47-LDA-DC vaccine (0.19 ± 0.04 vs. 0.36 ± 0.06; P = 0.03). In the latter group of mice, the numbers of $CD4^+CD25^+$ lymphocytes positive for intracellular FoxP3 expression ranged from 0.31 x 10^6 to 0.42 x 10^6 cells (Fig. 7C). Similar differences in frequencies of Treg cells were detected in the brachial lymph nodes and spleen of the immunized mice (data not shown). These results were consistent with the previously demonstrated differences in CCL22 expression levels between the LPS-matured 47-LDA-Fcγ2a$^+$ DCs and 47-LDA$^+$ DCs (Fig. 5C and D), and the reports that CCL22 represents the main DC-produced Treg-attracting chemokine [154, 206].

When CSFE-labeled $CD8^+$ T cells and Tregs prepared from 47-LDA-Fcγ2a-DC- and 47-LDA-DC-immunized mice were mixed at a 1:1 ratio and cocultured with the respective LPS-matured 47-LDA-Fcγ2a$^+$ DCs and 47-LDA$^+$ DCs for 72 h, both populations of Tregs mediated suppression of Teff cell expansion (Fig. 7B and D). However, the proliferation rate of Teff cells in 47-LDA-Fcγ2a$^+$ DC-stimulated cultures were significantly higher compared to stimulation elicited with 47-LDA$^+$ DCs (P = 0.02).

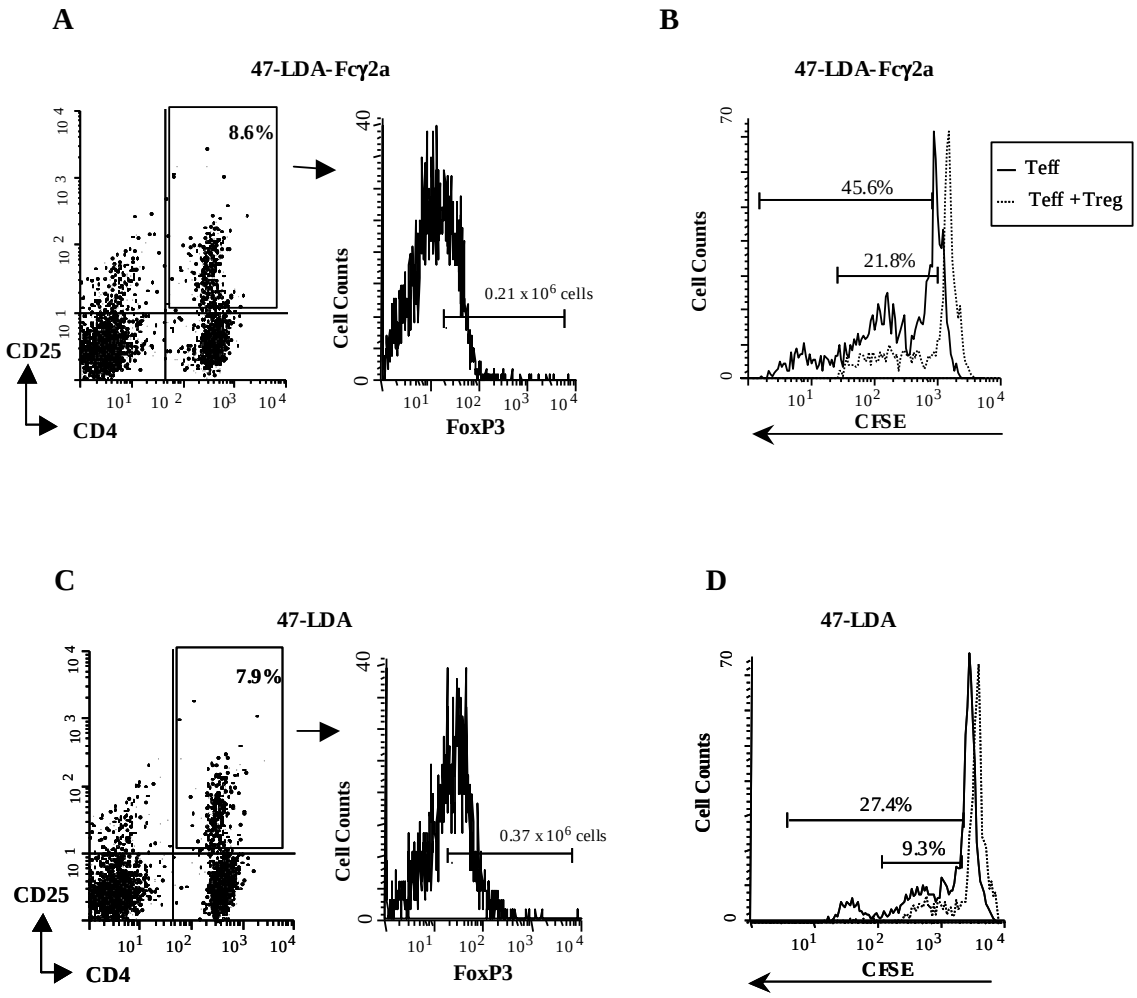


Figure 7. Analyses of Treg cell induction and function in mice immunized with 47-LDA-DC and 47-LDA-Fcγ2a-DC vaccines. Three weeks after the last immunization with 47-LDA-Fcγ2a-DCs (A) or 47-LDA-DCs (C), the axillary lymph nodes were removed and analyzed for Treg cell expression by staining with anti-CD4-PE, anti-CD25-FITC and anti-FoxP3-AlexaFluor 647 mAb, or the relevant isotype controls. The histograms illustrate the FoxP3 expression in the gated populations ($CD4^+CD25^+$ cells). To analyze the effect of Treg cells on Teff cell proliferation, $CD8^+$ cells from the 47-LDA-Fcγ2a-DC (B) or 47-LDA-DC (D) vaccine immunized mice were loaded with CFSE and cultured with 47-LDA-expressing syngeneic DCs (ratio 20:1) for 72 h in the presence or absence of Treg cells (ratio 1:1). Cells were stained with PE-conjugated anti-CD8 mAbs and analyzed by flow cytometry. The percentage of cells that underwent more than one round of cell division is indicated. Data are from one representative experiment of three performed.

5. Myeloablative total body irradiation in combination with the 47-LDA-Fcγ2a-DC vaccine enhances ATC therapy

In order to compare the therapeutic efficacy of the 47-LDA- and 47-LDA-Fcγ2a-DC vaccines, ACT experiments with antigen-experienced $CD8^+$ splenocytes in NXS2 tumor-bearing A/J mice (Fig. 2B) were performed. The $CD8^+$ T cells were delivered i.v. together with the 47-LDA- or 47-LDA-Fcγ2a-DC vaccine to NXS2 tumor-bearing mice that received a nonmyeloablative (5 Gy) or myeloablative (9 Gy) dose of TBI accompanied by a transplantation of syngeneic BM from naïve mice. Figure 8A shows that lymphodepletion with a nonmyeloablative (5 Gy) regimen prior to ACT and 47-LDA polypeptide-coated DC vaccine had a background inhibitory effect on tumor growth ($P = 0.28$). The antitumor efficacy of the adoptively transferred $CD8^+$ T cells was augmented after myeloablative (9 Gy) TBI, as determined by extension of the overall survival time from 25 to 70 days (Fig. 8A; $P = 0.007$). The myeloablative regimen was particularly effective in combination with adoptively transferred $CD8^+$ splenocytes stimulated with 47-LDA-Fcγ2a-DC vaccine (Fig. 8B). This treatment resulted in the complete remission of tumor growth in 22% of NXS2-bearing animals that received myeloablative dose of TBI accompanied by a transplantation of syngeneic BM from naïve mice ($P = 0.0003$). The therapy with $CD8^+$ T cells stimulated with 47-LDA-Fcγ2a-DC vaccine in the nonmyeloablative setting did not show complete tumor resolution, however, there was a significant prolongation of survival in the treated mice compared to the control group of animals (Fig. 8B; $P = 0.015$). The antitumor efficacy of the ACT was specific since all control mice developed progressively growing tumors and had to be sacrificed by day 25.

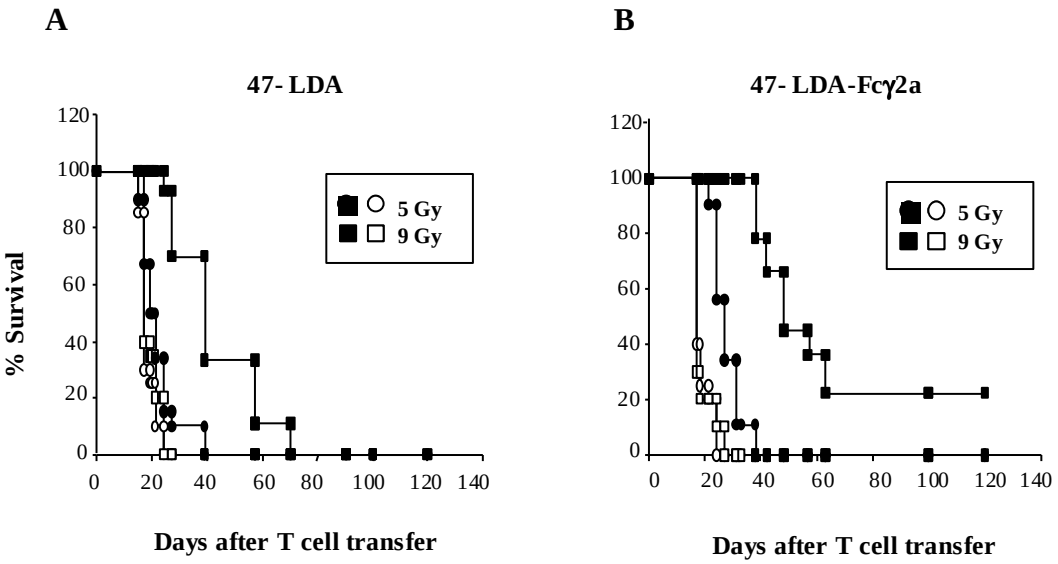


Figure 8. Inhibition of NXS2 primary tumor growth by ACT and 47-LDA-DC (A) and 47-LDA-Fcγ2a-DC (B) vaccines. A/J mice (n = 8 - 10) were injected s.c. with 2 x 10^6 NXS2 cells and treated 15 days later with i.v. adoptive transfer of $CD8^+$-enriched splenocytes isolated from 47-LDA- or 47-LDA-Fcγ2a-vaccinated syngeneic mice. Lymphopenia of tumor-bearing mice was induced by TBI (5 Gy;●) or (9 Gy;■) plus BM (10^7 cells) transplantation one day before $CD8^+$ T cell transfer as described in the *Materials and Methods* section. Mice were immunized in a 2-week interval with the DC vaccines in the presence of IL-15 and IL-21 vectors. NXS2 tumor-bearing mice that were irradiated with 5 Gy (○) or 9 Gy (□) plus BM transplant served as controls. Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method.

6. Control of metastatic disease by adoptively transferred CD8$^+$ splenocytes and 47-LDA-Fcγ2a-DC vaccine

The ability of NXS2 neuroblastoma to develop spontaneous metastases after excision of the primary tumor provided a model for investigating the efficacy of adoptively transferred CD8$^+$ splenocytes to control disseminated disease [207]. In view of the accumulating evidence that curative surgery in conjunction with depletion of Treg cells enables the development of long-lived tumor protection and CD8$^+$ T cell memory [160], the NXS2-*Rluc* tumor-bearing mice underwent nonmyeloablative (5 Gy) or myeloablative (9 Gy) TBI at the time of tumor excision and ACT of antigen-experienced CD8$^+$ splenocytes together with 47-LDA- or 47-LDA-Fcγ2a-DC vaccine. As shown in Fig. 9A, transfer of CD8$^+$ T cells expanded by 47-LDA polypeptide-coated DC delayed progression of the metastatic disease that was observed only in the myeloablated mice (P = 0.024). However, tumor-free survival was not observed in this group of animals. The antitumor efficacy of the adoptively transferred CD8$^+$ T cells was significantly enhanced by the ACT and 47-LDA-Fcγ2a-DC vaccination. As shown in Fig. 9B, only two of six NXS2-*RLuc*-challenged mice that received 5 Gy TBI prior to the ACT and 47-LDA-Fcγ2a-DC vaccine developed spontaneous metastases within a 50-day period of the treatment (P = 0.003). The remaining mice in this group exhibited a delay in progression of the metastatic disease with the longest survival time of 90 days. As expected, the highest antitumor effectiveness of the adoptively-transferred splenocytes with over 80% survival during a period of 120 days was observed after a myeloablative conditioning regimen used prior to the adoptive transfer of splenocytes from 47-LDA-Fcγ2a-immunized mice (Fig. 9B; P < 0.001). On the other hand, the control mice that had the primary tumor excised and received TBI had to be sacrificed by day 40 due to disease progression.

Protection against spontaneous metastases

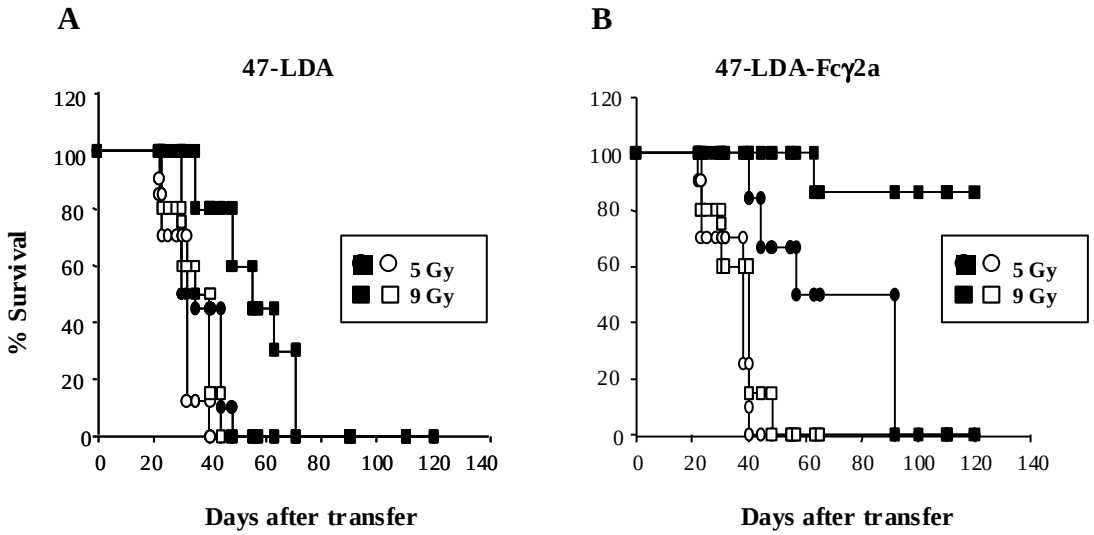
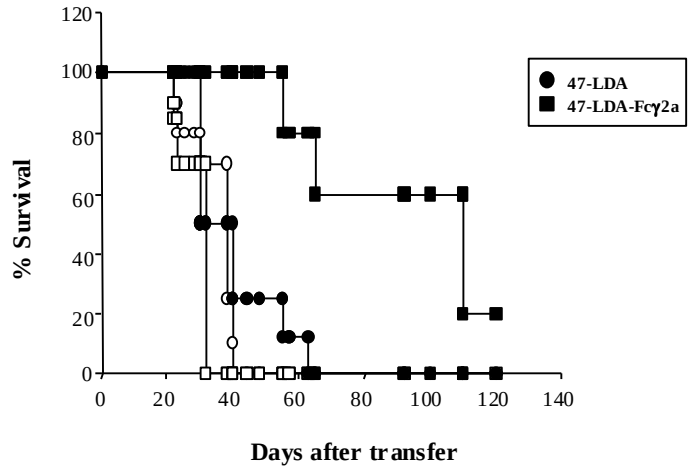


Figure 9. Control of metastatic disease by adoptively transferred $CD8^+$ splenocytes from immunized mice and 47-LDA-DC (A) and 47-LDA-Fcγ2a-DC (B) vaccines. For the immunotherapy of disseminated disease, NXS2-*RLuc*-bearing A/J mice that underwent nonmyeloablative (5 Gy;●) or myeloablative (9 Gy;■) TBI at the time of tumor excision received ACT of $CD8^+$ splenocytes (2 x 10^7 cells) from the immunized mice together with 47-LDA- or 47-LDA-Fcγ2a-DC vaccine. NXS2-*RLuc* challenged mice that were irradiated with 5 Gy (○) or 9 Gy (□) plus BM transplant served as controls. Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method.

7. Inhibition of metastatic disease by primary immune responses induced by the 47-LDA-Fcγ2a-DC vaccine

In the next set of experiments, it was examined whether the 47-LDA- and 47-LDA-Fcγ2a-DC vaccines had the ability to induce antitumor immune responses capable of inhibiting metastatic disease after adoptive transfer of naïve splenocytes to myeloablative mice after excision of the primary tumor. These experiments were performed using total splenocytes for ACT instead of $CD8^+$-enriched splenocytes as $CD4^+$ T cells are necessary for providing $CD8^+$ T cells with growth factors and can also mediate destruction of tumor cells [208]. Figure 10A shows that although the measurable therapeutic impact of the *in vivo* stimulated naïve T cells in NXS2-*Rluc*-bearing mice was lower compared to that mediated by the antigen-experienced counterparts, 20% treated mice remained tumor-free for over 100 days after ACT with 47-LDA-Fcγ2a-DC vaccine ($P = 0.007$). The 47-LDA-coated DCs were also capable of stimulating antitumor T cells responses, albeit to a smaller degree. The control mice that had the primary tumor excised with or without TBI and BM transplantation developed metastatic disease within the first 2 months of treatment, indicating a significant efficacy of the tumor-specific T cells induced by active immunization in tumor-bearing mice. The metastatic lesions developed primarily in the brachial and axillary lymph nodes and were confirmed by bioluminescence imaging (Fig. 10B)

A



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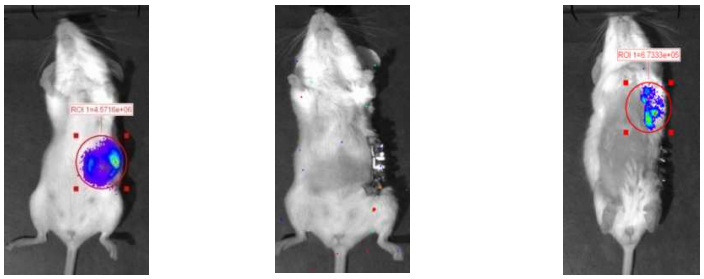


Primary tumor | 3 days after resection of primary tumor | 25 days after resection of primary tumor

Figure 10. Inhibition of NXS2-*RLuc* metastatic tumor growth by 47-LDA- and 47-LDA-Fcγ2a-DC vaccines and adoptive transfer of naïve splenocytes. (A) NXS2-*RLuc* tumor-bearing A/J mice that underwent myeloablative TBI at the time of tumor excision received ACT of splenocytes (2 x 10^7 cells) from naïve, syngeneic mice together with 47-LDA- or 47-LDA-Fcγ2a-DC vaccine. Mice were immunized in a 2-week interval with the DC vaccine in the presence of IL-15 and IL-21 vectors. The control mice had the primary tumor excised with (○) or without (□) TBI and BM transplantation. Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method. (B) Detection of NXS2-*RLuc* spontaneous metastases after resection of primary tumor. A/J mice had the primary NXS2-*Rluc* tumor resected and were monitored for metastasis every 3-5 days under IVIS 50 Imaging System. *In vivo* images of representative mouse with the metastasis to axillary and brachial lymph nodes are shown.

8. Cellular responses in tumor-bearing and tumor-free mice after ACT and DC vaccination

The ability of therapeutic 47-LDA-Fcγ2a- and 47-LDA-DC vaccines to expand Teff cells was investigated during ACT of naïve splenocytes to myeloablated mice after excision of the primary NXS2-*Rluc* tumor. As the control mice or those receiving adoptively transferred splenocytes together with the 47-LDA-DC vaccine developed metastatic disease that frequently localized in the brachial and axillary lymph nodes, animals with visible metastatic lesions were examined for antitumor immune responses in the spleen. It was observed that less than 15% of $CD4^+$ or $CD8^+$ splenocytes in the tumor-bearing mice underwent *in vitro* division after 72-h stimulation with 47-LDA-expressing DCs (Fig. 11A), and the CTL responses to NXS2 cells were at a background level in the tumor-bearing mice (Fig. 11B). In contrast, the same analysis performed in mice that received ACT and 47-LDA-Fcγ2a-DC vaccine but had no sign of metastatic disease revealed that 66.6 ± 11% $CD4^+$ and 44.3 ± 7.5 % $CD8^+$ splenocytes underwent cell division in cultures stimulated with 47-LDA-expressing DC (Fig. 11C), and the proliferative responses were associated with antitumor CTL activities against parental NXS2 cells (Fig. 11D).

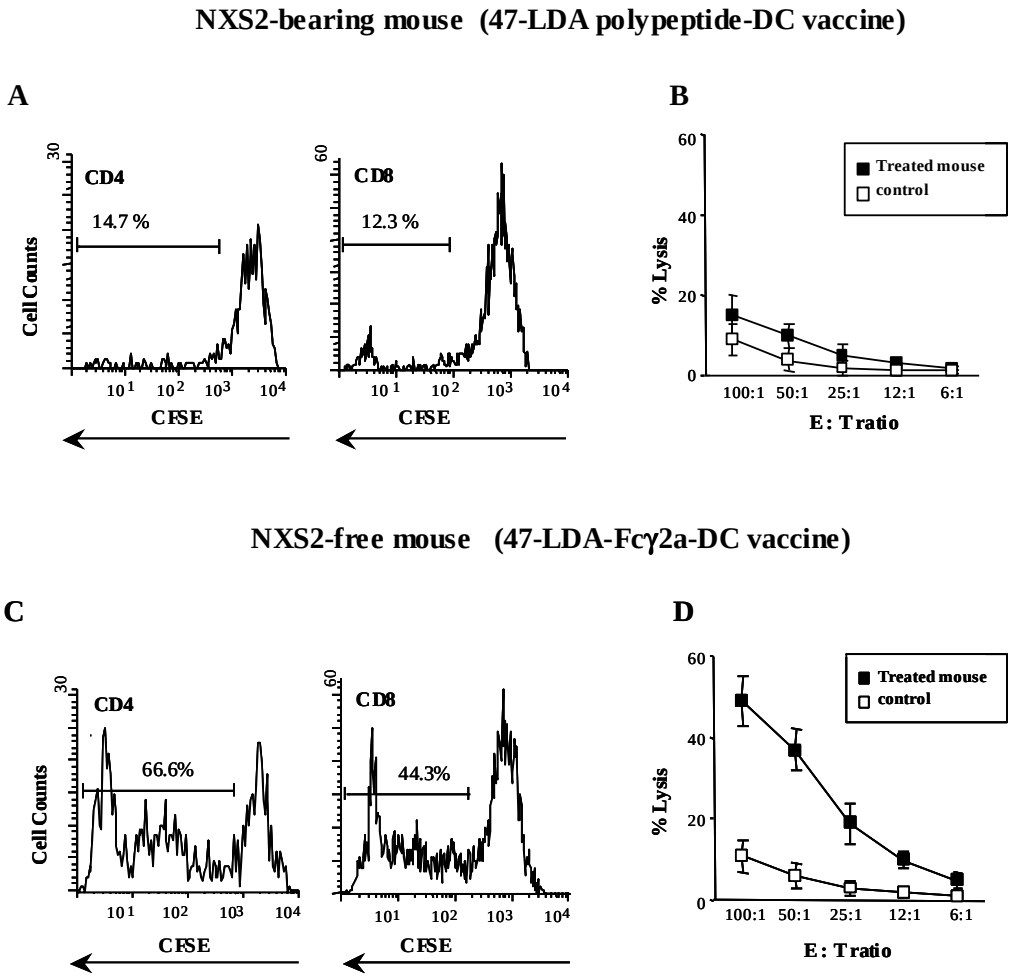
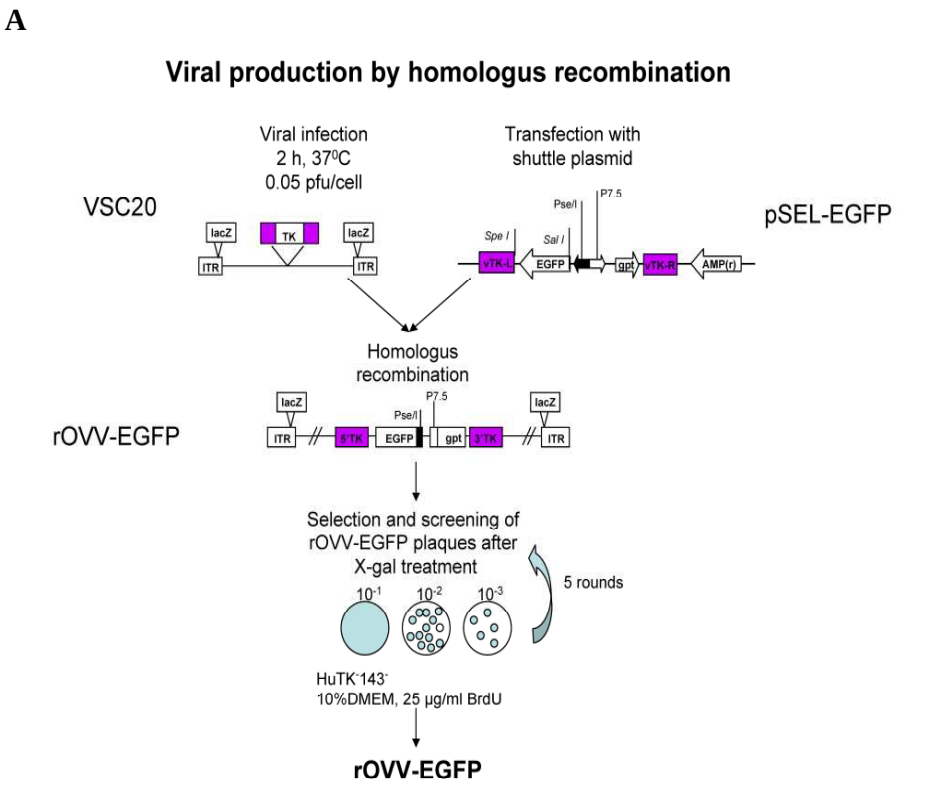


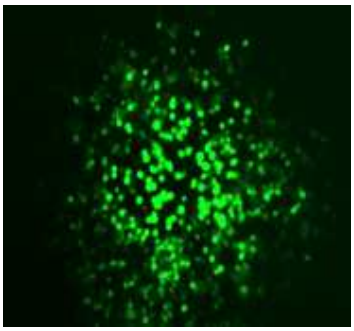
Figure 11. Analyses of cellular responses in tumor-bearing and tumor-free mice after ACT and DC vaccination. *Upper panel*, Lack of proliferative responses (A) and CTL activity (B) in splenocytes of NXS2-bearing mice. (A) Splenocytes isolated from mice that developed progressively growing tumor after ACT of naïve splenocytes and 47-LDA polypeptide-DC vaccine were loaded with CFSE and cultured with 47-LDA-expressing syngeneic DC (ratio 20:1) for 72 h. Cells were stained with PE-conjugated anti-CD4 or anti-CD8 mAbs and analyzed by flow cytometry. The percentage of cells that underwent more than one round of cell division is indicated. Data are from one representative experiment of three performed. (B) CTL activities of stimulated or control $CD8^+$ splenocytes from tumor-bearing mice against NXS2 cells were analyzed in a standard ^{51}Cr-release assay. All determinations were made in triplicate samples, and results are presented as the means ± SD of three independent experiments. *Lower panel*, Analyses of proliferative responses (C) and CTL activities against NXS2 (D) in tumor-free mice after receiving ACT from naïve mice and 47-LDA-Fcγ2a-DC vaccine. Experiments were carried out as described in the upper panel.

9. Generation of rOVV expressing enhanced green fluorescence protein (EGFP) and the 47-LDA-Fcγ2a fusion protein

In order to determine that the antitumor effect of 47-LDA-Fcγ2a-DC vaccine is not limited to the ACT in myeloablative tumor-bearing hosts, 47-LDA-Fcγ2a fusion protein was expressed in rOVV and delivered to NXS2-bearing A/J mice.

The rOVV vaccinia virus expressing EGFP and 47-LDA-Fcγ2a fusion protein was generated by homologous recombination in CV-1 cells using the VSC20 vaccinia virus and the vaccinia shuttle plasmids pSEL-EGFP and pCB023-47-LDA-Fcγ2a, respectively (Fig 12A). The rOVV-EGFP served as a specificity control for *in vivo* experiments and to determine its ability to function as an oncolytic virus. The pCB023-47-LDA-Fcγ2a shuttle plasmid was generated by cloning the 47-LDA-Fcγ2a fusion protein gene from pFUSE-mIgG2Aa-Fc1 vector into the *EcoRI* and *SmaI* restriction enzyme sites of pCB023 plasmid. Multiple plaques of the recombinant viruses were selected in TK^- cells by BrdU selection as described [209]. The expression of EGFP in rOVV-EGFP-infected NXS2 cells was confirmed by immunofluorescence microscopy (Fig. 12B), whereas secretion of the 47-LDA-Fcγ2a fusion protein from rOVV-47-LDA-Fcγ2a-infected NXS2 cells was determined by immunoblotting with 14G2a mAb (Fig. 12C).





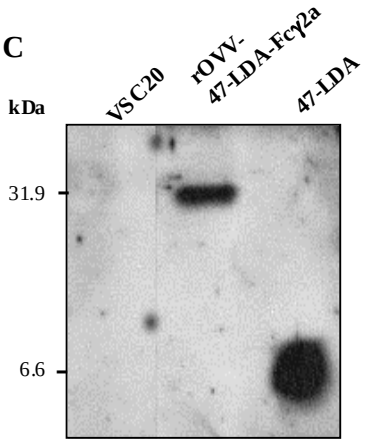


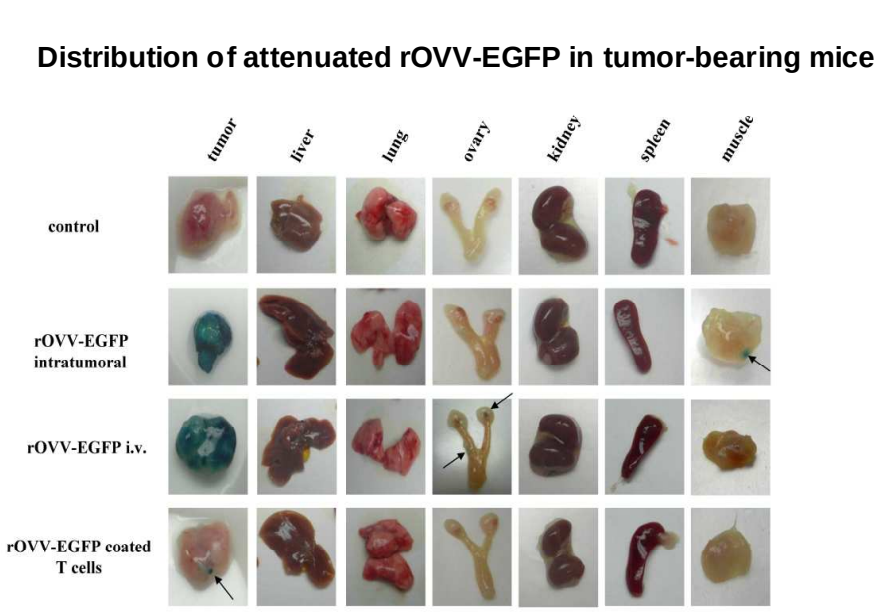
Figure 12. Generation of rOVV-EGFP and rOVV-47-LDA-Fcγ2a. (A) Schematic representation of homologous recombinantion between VSC20 vaccinia virus and the vaccinia shuttle plasmids pSEL-EGFP. A similar approach with vaccinia shuttle plasmids pCB023-47-LDA-Fcγ2a was used to generate rOVV-47-LDA-Fcγ2a. (B) The NXS2 cells were infected with 10^8 pfu of rOVV-EGFP and analyzed for expression of EGFP by immunofluorescence microscopy. (C) Secretion of 47-LDA-Fcγ2a fusion protein from rOVV-47-LDA-Fcγ2a-infected NXS2 cells was confirmed by Western blotting of culture supernatant with biotinylated 14G2a mAb and streptavidin-HRP. 47-LDA polypeptide was included as a positive control.

10. *In vivo* pathogenicity and biodistribution of rOVV-EGFP in A/J mice

In order to determine the pathogenicity of rOVV-EGFP, the A/J mice were injected with different doses of virus ranging from 10^7 to 10^9 pfu. The dose of 10^8 pfu was chosen for *in vivo* treatment because in this amount the mutant virus did not exhibit any visible signs of pathogenicity in mice. This was in contrast to the effect observed with the wild-type vaccinia virus expressing β-gal gene (vSC8), which killed mice within 3-4 days. The rOVV-EGFP virus was not pathogenic in immunocompromised mice, which is consistent with studies by Dr. Bartlett's group with TK^-, VGF^- double mutant (vvDD) in nude mice [180].

To investigate the biodistribution of rOVV-EGFP, NXS2 tumor-bearing A/J mice received i.v or intratumoral injection of 10^8 pfu of rOVV-EGFP. In some experiments, adoptive transfer of NXS2-specific $CD8^+$ T cells (10^7 cells) loaded with rOVV-EGFP was used to chaperone the virus and deliver it to the tumor site. At day 4th following viral administration, samples of normal tissues and tumor were harvested and examined for the presence of rOVV-EGFP by staining with X-Gal. The level of virus in all samples was titered on TK^- cells. Data in Fig. 13A and B provide a comparison of rOVV-EGFP biodistribution in tumor and normal tissues after different routes of delivery. As expected, intratumoral delivery of rOVV-EGFP induced shrinkage of the tumor mass with a little or none virus present in other tissues except for a low titer ($0.5\log_{10}$ PFU) in muscle adjacent to the tumor. The i.v. delivery of the virus resulted in the highest viral yield in the tumor ($5.7\log_{10}$ PFU) and ~1,000-fold lower level in ovaries ($2.1\log_{10}$ PFU), which is the organ preferably targeted by vaccinia virus. The least efficient was the adoptive transfer of T lymphocytes coated with rOVV-EGFP *in vitro*, which only resulted in 3.7 $\log_{10}$ PFU of the virus in tumor cells. Altogether, these results demonstrated that the yield of rOVV-EGFP in normal tissues was markedly lower than in NXS2 tumor cells regardless of the route of viral delivery, confirming the safety and tumor specificity of oncolytic vaccinia virus.

A



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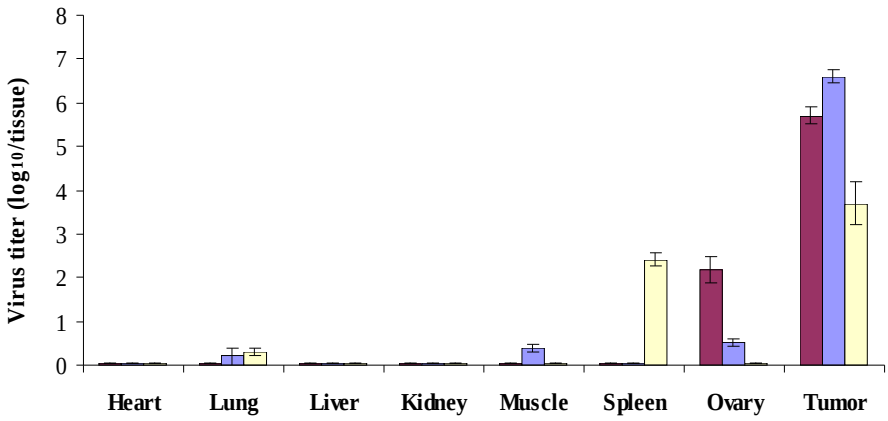

Figure 13. *In vivo* biodistribution of rOVV-EGFP in NXS2 tumor-bearing mice. (A) X-gal staining of organs. A/J mice were challenged with $2x10^6$ NXS2 cells. At day 14 after implantation, 10^8 pfu of rOVV-EGFP was injected intratumoral or i.v. One group of mice received i.v. injection of antigen-specific T lymphocytes coated with rOVV-EGFP *in vitro*. Animals were sacrificed after 4 days and organs were harvested, washed in PBS and stained with X-gal for 15 minutes. (B) The samples were analyzed for the presence of rOVV-EGFP by PFU assay on TK^- cells. The harvested organs were homogenized and used for infection of TK^- cells. Bars: purple (i.v.); blue (intratumoral); yellow (rOVV-EGFP-coated T cells).

11. Virotherapy-based cancer vaccine with rOVV expressing 47-LDA-Fcγ2a fusion protein

The therapeutic efficacy of the 47-LDA-Fcγ2a fusion protein expressed in the rOVV vaccinia virus was examined in NXS2 tumor-bearing A/J mice. Because the oncolytic TK^-, VGF^- mutant of vaccinia rOVV-47-LDA-Fcγ2a exhibits tumor-specific replication, it was anticipated that the fusion protein will be secreted from infected cells for crosspresentation by tumor infiltrating DCs at the site of oncolysis [180, 210]. Two weeks after tumor challenge, A/J mice received i.v. injection of 10^8 pfu of rOVV-47-LDA-Fcγ2a. In parallel, another group of mice was injected with the rOVV-EGFP virus as a specificity control to determine the extent of protection elicited by TAA released from virally-infected cells. Animals were examined for tumor growth by measuring s.c. tumors once to thrice a week. As shown in Fig. 14A, approximately 25% of NXS2-bearing mice that were treated with the rOVV-EGFP virus became tumor-free, whereas PBS-treated control mice developed progressively growing tumors and had to be sacrificed by day 20. The highest antitumor effectiveness characterized by over 60% survival during a period of 100 days, was observed in tumor-bearing mice after the vOVV-47-LDA-Fcγ2a oncolytic immunovirotherapy ($P < 0.001$).

We next investigated whether the oncolytic virotherapy treatment was capable of facilitating tumor-protective immune memory in tumor-free mice. NXS2-challenged mice ($n = 6$), which remained free of tumor for at least 40 days after rOVV-EGFP or rOVV-47-LDA-Fcγ2a treatment, were re-challenged s.c. with 10^6 NXS2 cells. Figure 14B shows that all rOVV-EGFP-treated and NXS2 tumor re-challenged mice developed progressively growing tumor. However, we observed a 20-day delay in the initiation of tumor growth in the rOVV-EGFP-treated mice compared to control counterparts, consistent with the ability of rOVV to induce antitumor immune responses due to uptake of apoptotic cells by DCs and cross-presentation of TAAs during oncolytic virotherapy [175]. On the other hand, only one of six rOVV-47-LDA-Fcγ2a-treated and re-challenged mice developed slowly progressing tumor 35 days after the re-challenge, reflecting the significant antitumor influence of the 47-LDA-Fcγ2a-induced immunity compared to rOVV-EGFP- or PBS-treated mice (Fig. 14B, $P < 0.001$).

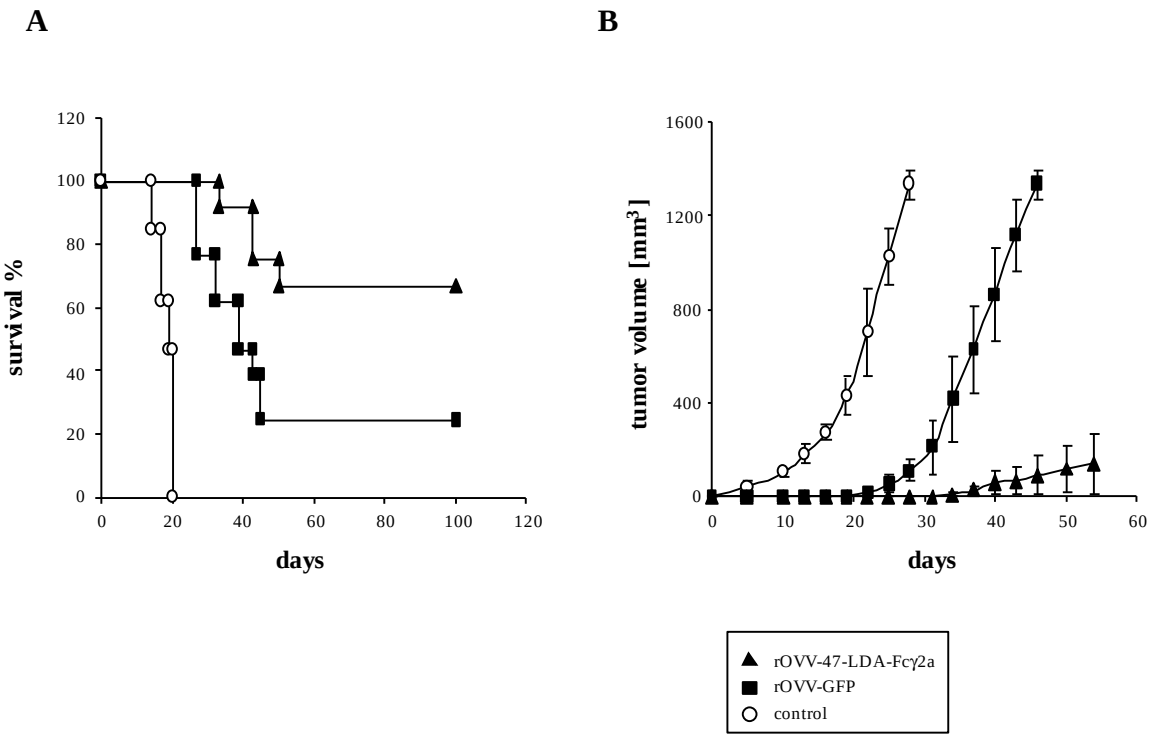

Figure 14. Therapeutic oncolytic virotherapy-based cancer vaccine with rOVV-EGFP or rOVV-47-LDA-Fcγ2a fusion protein. (A) Inhibition of tumor growth by rOVV-47-LDA-Fcγ2a vector. A/J mice (n = 10) were injected s.c. with 2 x 10^6 NXS2 cells and treated 15 days later with i.v. injection of 10^8 PFU of rOVV-47-LDA-Fcγ2a (▲) or rOVV-EGFP(■) vector. Tumor-bearing mice that were treated with PBS served as controls (○). Survival was defined as the point at which mice were sacrificed due to extensive tumor growth. Kaplan-Meier survival plots were prepared, and significance was determined using logrank Mantel-Cox method. (B) Tumor-specific immune memory protected mice from NXS2 re-challenge. Tumor-free mice after treatment with rOVV-47-LDA-Fcγ2a (▲) or rOVV-EGFP (■) vector were re-challenged with NXS2 cells. Control mice were challenged with NXS2 tumor only (○). Animals were examined daily until the tumor became palpable, after which tumor growth was monitored by measuring s.c. tumors once to thrice a week.

VIII. DISCUSSION

Successful induction of antitumor immunity by cancer vaccine is associated with the induction of functional cytotoxic T cells and reduced expansion of Treg cells. To develop a means of limiting the ability of cancer vaccines to interact with Treg cells in tumor-bearing mice, we first compared the generation of functional tumor-specific cellular responses by the 47-LDA peptide mimic presented to DCs either as a linear polypeptide in conjunction with universal Th epitopes or as a fusion protein with the murine IgG2a Fc fragment. It was shown that the 47-LDA-Fcγ2a fusion protein-DC vaccine not only expanded tumor-specific $CD8^+$ T cells, but also induced primary immune responses after ACT of naïve splenocytes to tumor-bearing mice that underwent myeloablative TBI and BM transplantation (Fig. 10A). The 47-LDA-Fcγ2a vaccine-induced $CD8^+$ T cells inhibited growth of the primary tumors as well as protected the tumor-bearing mice from the development of minimal residual disease (Fig. 8B and 9B). On the other hand, protection elicited by the 47-LDA polypeptide-DC vaccine resulted in a delay of tumor growth, which was significant only after ACT in the myeloablative setting (Fig. 8A and 9A). The results of these studies are consistent with the findings that FcγRs are capable of modulating T cell-mediated immune responses during therapeutic vaccination in tumor-bearing mice [211-214].

The mechanisms responsible for the increased ability of 47-LDA-Fcγ$2a^+$ DCs to induce higher levels of tumor-specific immune responses compared to those generated by the 47-LDA^+ DCs remain to be elucidated. It is possible that the elevated number of IL-12-expressing cells within the 47-LDA-Fcγ$2a^+$ DC population compared to the 47-LDA^+ counterpart after LPS-induced maturation contributed to this effect since antigen-presenting cells that express IL-12 have augmented capacity to prime type 1 rather than Treg cell responses [154, 215]. This observation is further supported by the reduced expression of CCL22 chemokine in 47-LDA-Fcγ$2a^+$ DCs (Fig. 5D and C) and lower proportion of Treg cells in 47-LDA-Fcγ$2a^+$ DC-immunized mice compared to animals that received the 47-LDA-DC vaccine (Fig. 7A and C). Our results are consistent with the previous findings that the character of the inflammatory environment can affect the balance between Teff and Treg cell activation by instructing the maturing DCs to adopt a stable propensity to interact with each of these T-cell types [154, 209, 215-217]. It is noteworthy however that although alteration of DC functions has been previously

suggested in the design of a cancer vaccine [214, 218], tuning of DC responses to counteract the immunosuppression associated with tumor progression [219-222] during therapeutic vaccination with a mimotope of tumor-associated antigens has not been extensively evaluated. Thus, these findings highlight a new application of peptide mimotopes for immunotherapy of cancer.

It is well established that activating and inhibitory low-affinity FcγRs are critical for the modulation of effector immune responses [204]. Their role in the induction of adaptive immunity showed that alteration in the function of activating/inhibitory receptors during administration of antigens to DCs in a form of immune complexes can influence activation of DCs *in vivo* and enhance antitumor T-cell responses [212]. This notion is supported by the observation that selective blockade of inhibitory FcγRIIb receptor enables human DC maturation and immunity to antibody-coated tumor cells [171, 172], and DCs from FcγRIIb-deficient mice show higher expression of co-stimulatory molecules which could account for an increased capacity to prime antigen-specific T cells [223]. However, the use of large protein carriers creates difficulties in terms of reproducibility of the antigen binding that may endanger vaccine effectiveness and/or practical feasibility. Therefore, synthetic constructs encompassing the antigenic and helper epitopes as well as the Fc portion of IgG antibody with increased binding affinity to activating FcγRs should offer distinct advantages over immune complexes in terms of manufacturing and characterization. This also suggests that the ability of 47-LDA-Fcγ2a fusion protein to empower DCs to efficiently expand and prime specific $CD8^+$ CTL responses in tumor-bearing mice might lead to improved efficacy of tumor immunotherapy.

The study of antitumor immune responses after DC vaccines or surgery has shown that Treg cells are a fundamental obstacle to the development of T-cell memory in hosts bearing poorly immunogenic tumors [154, 160]. Although surgery currently remains the leading cure for solid tumors, memory T-cell responses may be required for the durable prevention of tumor recurrence and metastasis following surgery. As seen in most patients with cancer, our results showed that surgery or DC vaccine alone does not induce protection against poorly immunogenic tumor-associated antigens. However, in our model, surgery in combination with lymphodepletion, ACT and DC vaccination enables the development of long-lived tumor protection. Although we cannot exclude the possibility that the tumor itself served as the source of antigen, presumably priming

T-cell responses against multitude of tumor antigens during induction of postsurgical immunity in the absence of Tregs, [224-226], the antitumor immunity generated in the absence of the 47-LDA mimotope DC vaccination only extended the survival of tumor-bearing mice. In contrast, active immunization with 47-LDA-Fcγ2a fusion protein targeted to the activating FcγRs in a myeloablated tumor-bearing host led to a significant suppression of primary tumor and protection from the development of spontaneous metastatic disease.

While the use of *ex vivo* generated DCs provides a unique opportunity to avoid tumor-induced DC dysfunction and allows for precise manipulation of DC properties, the need for specialized cell culture facilities for the *ex vivo* manipulation of patient's cells prompted attempts to develop cell-free vaccines capable of targeting endogenous DCs within the bodies of tumor-bearing hosts. Such vaccines engineered to deliver tumor antigens or their mimotopes selectively to DCs can be coupled with strategies to induce DC polarization *in vivo* without any *ex vivo* treatment.

Recently, oncolytic virotherapy has attracted a lot of attention as a novel approach capable of destructing cancer cells due to selective and efficient amplification of the virus in transformed cells. Moreover, viruses containing tumor associated genes represent an attractive vector to induce immune responses to weak immunogens in cancer immunotherapy protocols. The obtained results using the virotherapy approach with rOVV-47-LDA-Fcγ2a vector, which delivers the transgene directly to tumor lesions for secretion and crosspresentation by DCs, demonstrated the induction of protective Teff as well as memory T cells in the absence of any exogenous cytokine treatment, ACT, TBI, or *ex vivo* manipulation (Fig. 14A and B). Further investigation is required to better define the effect of crosspresentation of the virally-delivered 47-LDA-Fcγ2a fusion protein in the tumor microenvironment on the balance between Teffs and Tregs as well as the effectiveness and longevity of the virotherapy-induced antitumor immune responses. In this regard, a growing body of evidence indicate that VV elicits innate immune responses through the TLR2/MyD88-dependent pathway, resulting in the production of proinflammatory cytokines, and a TLR-independent pathway, leading to the activation of IFN-γ *in vitro* and *in vivo* [195]. Because sustained stimulation of TLRs of the innate immunity is required for breaking established Treg-mediated tolerance *in vivo* and VV can provide TLR signals, this unique potency of rOVV as a vaccine vehicle can lead to activation of host defense and protect the mice from tumor

challenge [194, 196]. Thus, the possibility of breaking of CD8 tolerance by virus-based vaccines in the presence of $CD4^+CD25^+$ Tregs suggests that rOVV expressing the 47-LDA-Fcγ2a fusion protein or other tumor-associated antigens may prove an appealing alternative to DC vaccines for overcoming $CD4^+CD25^+$ T cell-mediated CD8 tolerance *in vivo*.

In summary, this work emphasises the importance of exploring the uptake and processing of tumor/self-antigens by DCs to activate immune effector cells and limit the ability of attracting anti-inflammatory Treg cells. Our findings illuminate a new paradigm for cancer immunotherapies aimed at the selective activation of the inflammatory versus regulatory type of immune cells.

IX. FUTURE PLANS

It has been demonstrated that targeting activating FcγRs on DCs with 47-LDA-Fcγ2a fusion protein led to increased generation of tumor-specific Teff cells and limited induction of Tregs. The antitumor effect of the 47-LDA-Fcγ2a fusion protein was observed not only during the adoptive transfer of antigen-experienced or naïve T cells and DC vaccination but also when 47-LDA-Fcγ2a transgene was delivered to the tumor-bearing mice by the rOVV vector. Further research is required to evaluate the effect of crosspresentation of the virally-delivered 47-LDA-Fcγ2a fusion protein in the tumor microenvironment on the balance between Teffs and Tregs. Moreover, additional strategies to augment the outcome of rOVV-47-LDA-Fcγ2a virotherapy-based cancer vaccine should also be explored:

1. Since the efficacy or antitumor immunity of current virotherapy is largely limited by the extent of viral replication within the tumors, novel approaches need to be explored to modify the tumor microenvironment and augment spreading infection. In this regard, targeting tumor microenvironment with a tumor vascular disrupting agents or coadministration of enzymes to degrade the extracellular milieu may enhance viral load in tumor lesions and augment antitumor efficacy.
2. The presence of neutralizing antibodies to viral vectors represents a major issue that can limit the application of virotherapy in cancer patients. Methods for shielding the rOVV from neutralizing antibodies using nanoparticles or infected cells as carriers may increase the viral resistance and allowed for repeated immunizations.
3. Combination treatment of the rOVV with chemotherapeutic agents may represent a novel treatment platform in which new findings in the laboratory can be translated into the clinic.

X. REFERENCES

1 Coley, W. B., The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin Orthop Relat Res* 1991: 3-11.

2 Gromet, M. A., Epstein, W. L. and Blois, M. S., The regressing thin malignant melanoma: a distinctive lesion with metastatic potential. *Cancer* 1978. 42: 2282-2292.

3 Gross, L., Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res* 1943. 3: 326-333.

4 Prehn, R. T. and Main, J. M., Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 1957. 18: 769-778.

5 Foley, E. J., Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res* 1953. 13: 835-837.

6 Klein, G., Sjogren, H. O., Klein, E. and Hellstrom, K. E., Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res* 1960. 20: 1561-1572.

7 Burnet, M., Cancer; a biological approach. I. The processes of control. *Br Med J* 1957. 1: 779-786.

8 Zou, W., Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005. 5: 263-274.

9 Stutman, O., Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science* 1974. 183: 534-536.

10 Outzen, H. C., Custer, R. P., Eaton, G. J. and Prehn, R. T., Spontaneous and induced tumor incidence in germfree "nude" mice. *J Reticuloendothel Soc* 1975. 17: 1-9.

11 Rygaard, J. and Povlsen, C. O., The mouse mutant nude does not develop spontaneous tumours. An argument against immunological surveillance. *Acta Pathol Microbiol Scand B Microbiol Immunol* 1974. 82: 99-106.

12 Hewitt, H. B., Blake, E. R. and Walder, A. S., A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. *Br J Cancer* 1976. 33: 241-259.

13 Van Pel, A. and Boon, T., Protection against a nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis. *Proc Natl Acad Sci U S A* 1982. 79: 4718-4722.

14 Pardoll, D., Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 2003. 21: 807-839.

15 Hunig, T. and Bevan, M. J., Specificity of cytotoxic T cells from athymic mice. *J Exp Med* 1980. 152: 688-702.

16 Ikehara, S., Pahwa, R. N., Fernandes, G., Hansen, C. T. and Good, R. A., Functional T cells in athymic nude mice. *Proc Natl Acad Sci U S A* 1984. 81: 886-888.

17 Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J. and Schreiber, R. D., IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 2001. 410: 1107-1111.

18 Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. and Schreiber, R. D., Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002. 3: 991-998.

19 Melief, C. J., Toes, R. E., Medema, J. P., van der Burg, S. H., Ossendorp, F. and Offringa, R., Strategies for immunotherapy of cancer. *Adv Immunol* 2000. 75: 235-282.

20 Santin, A. D., Hermonat, P. L., Ravaggi, A., Bellone, S., Roman, J. J., Smith, C. V., Pecorelli, S., Radominska-Pandya, A., Cannon, M. J. and Parham, G. P., Phenotypic and functional analysis of tumor-infiltrating lymphocytes compared with tumor-associated lymphocytes from ascitic fluid and peripheral blood lymphocytes in patients with advanced ovarian cancer. *Gynecol Obstet Invest* 2001. 51: 254-261.

21 Baxevanis, C. N., Dedoussis, G. V., Papadopoulos, N. G., Missitzis, I., Stathopoulos, G. P. and Papamichail, M., Tumor specific cytolysis by tumor infiltrating lymphocytes in breast cancer. *Cancer* 1994. 74: 1275-1282.

22 Itoh, K., Platsoucas, C. D. and Balch, C. M., Autologous tumor-specific cytotoxic T lymphocytes in the infiltrate of human metastatic melanomas. Activation by interleukin 2 and autologous tumor cells, and involvement of the T cell receptor. *J Exp Med* 1988. 168: 1419-1441.

23 Muul, L. M., Spiess, P. J., Director, E. P. and Rosenberg, S. A., Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *J Immunol* 1987. 138: 989-995.

24 Schumacher, K., Haensch, W., Roefzaad, C. and Schlag, P. M., Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. *Cancer Res* 2001. 61: 3932-3936.

25 Marrogi, A. J., Munshi, A., Merogi, A. J., Ohadike, Y., El-Habashi, A., Marrogi, O. L. and Freeman, S. M., Study of tumor infiltrating lymphocytes and transforming growth factor-beta as prognostic factors in breast carcinoma. *Int J Cancer* 1997. 74: 492-501.

26 Vesalainen, S., Lipponen, P., Talja, M. and Syrjanen, K., Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. *Eur J Cancer* 1994. 30A: 1797-1803.

27 Halpern, A. C. and Schuchter, L. M., Prognostic models in melanoma. *Semin Oncol* 1997. 24: S2-7.

28 Naito, Y., Saito, K., Shiiba, K., Ohuchi, A., Saigenji, K., Nagura, H. and Ohtani, H., CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998. 58: 3491-3494.

29 Dougan, M. and Dranoff, G., Immune therapy for cancer. *Annu Rev Immunol* 2009. 27: 83-117.

30 Reilly, R. T., Machiels, J. P., Emens, L. A., Ercolini, A. M., Okoye, F. I., Lei, R. Y., Weintraub, D. and Jaffee, E. M., The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res* 2001. 61: 880-883.

31 Yu, Z. and Restifo, N. P., Cancer vaccines: progress reveals new complexities. *J Clin Invest* 2002. 110: 289-294.

32 Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H. and Beach, D., A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* 1995. 269: 1281-1284.

33 Lugo, T. G., Pendergast, A. M., Muller, A. J. and Witte, O. N., Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* 1990. 247: 1079-1082.

34 Barbacid, M., ras genes. *Annu Rev Biochem* 1987. 56: 779-827.

35 Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C., p53 mutations in human cancers. *Science* 1991. 253: 49-53.

36 Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H. and White, D. E., Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 1998. 4: 321-327.

37 Godelaine, D., Carrasco, J., Lucas, S., Karanikas, V., Schuler-Thurner, B., Coulie, P. G., Schuler, G., Boon, T. and Van Pel, A., Polyclonal CTL responses observed in melanoma patients vaccinated with dendritic cells pulsed with a MAGE-3.A1 peptide. *J Immunol* 2003. 171: 4893-4897.

38 Gilboa, E., The risk of autoimmunity associated with tumor immunotherapy. *Nat Immunol* 2001. 2: 789-792.

39 Overwijk, W. W., Lee, D. S., Surman, D. R., Irvine, K. R., Touloukian, C. E., Chan, C. C., Carroll, M. W., Moss, B., Rosenberg, S. A. and Restifo, N. P., Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. *Proc Natl Acad Sci U S A* 1999. 96: 2982-2987.

40 Ludewig, B., Ochsenbein, A. F., Odermatt, B., Paulin, D., Hengartner, H. and Zinkernagel, R. M., Immunotherapy with dendritic cells directed against tumor antigens shared with normal host cells results in severe autoimmune disease. *J Exp Med* 2000. 191: 795-804.

41 Pardoll, D. M., Inducing autoimmune disease to treat cancer. *Proc Natl Acad Sci U S A* 1999. 96: 5340-5342.

42 Caspi, R. R., Immunotherapy of autoimmunity and cancer: the penalty for success. *Nat Rev Immunol* 2008. 8: 970-976.

43 Lane, C., Leitch, J., Tan, X., Hadjati, J., Bramson, J. L. and Wan, Y., Vaccination-induced autoimmune vitiligo is a consequence of secondary trauma to the skin. *Cancer Res* 2004. 64: 1509-1514.

44 Bronte, V., Apolloni, E., Ronca, R., Zamboni, P., Overwijk, W. W., Surman, D. R., Restifo, N. P. and Zanovello, P., Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res* 2000. 60: 253-258.

45 Sell, S., Cancer-associated carbohydrates identified by monoclonal antibodies. *Hum Pathol* 1990. 21: 1003-1019.

46 Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L. and Kerbel, R. S., Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* 1987. 236: 582-585.

47 Hakomori, S., Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res* 1996. 56: 5309-5318.

48 Matsuura, H., Takio, K., Titani, K., Greene, T., Levery, S. B., Salyan, M. E. and Hakomori, S., The oncofetal structure of human fibronectin defined by monoclonal antibody FDC-6. Unique structural requirement for the antigenic specificity provided by a glycosylhexapeptide. *J Biol Chem* 1988. 263: 3314-3322.

49 Muramatsu, T., Carbohydrate signals in metastasis and prognosis of human carcinomas. *Glycobiology* 1993. 3: 291-296.

50 Hakomori, S., Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv Cancer Res* 1989. 52: 257-331.

51 Livingston, P. O., Zhang, S. and Lloyd, K. O., Carbohydrate vaccines that induce antibodies against cancer. 1. Rationale. *Cancer Immunol Immunother* 1997. 45: 1-9.

52 Monzavi-Karbassi, B., Pashov, A., Jousheghany, F., Artaud, C. and Kieber-Emmons, T., Evaluating strategies to enhance the anti-tumor immune response to a carbohydrate mimetic peptide vaccine. *Int J Mol Med* 2006. 17: 1045-1052.

53 Monzavi-Karbassi, B., Cunto-Amesty, G., Luo, P. and Kieber-Emmons, T., Peptide mimotopes as surrogate antigens of carbohydrates in vaccine discovery. *Trends Biotechnol* 2002. 20: 207-214.

54 Kieber-Emmons, T., Monzavi-Karbassi, B., Wang, B., Luo, P. and Weiner, D. B., Cutting edge: DNA immunization with minigenes of carbohydrate mimotopes induce functional anti-carbohydrate antibody response. *J Immunol* 2000. 165: 623-627.

55 Kieber-Emmons, T., Luo, P., Qiu, J., Chang, T. Y., O, I., Blaszczyk-Thurin, M. and Steplewski, Z., Vaccination with carbohydrate peptide mimotopes promotes anti-tumor responses. *Nat Biotechnol* 1999. 17: 660-665.

56 O, I., Kieber-Emmons, T., Otvos, L., Jr. and Blaszczyk-Thurin, M., Peptides mimicking sialyl-Lewis A isolated from a random peptide library and peptide array. *Ann N Y Acad Sci* 1999. 886: 276-279.

57 Bolesta, E., Kowalczyk, A., Wierzbicki, A., Rotkiewicz, P., Bambach, B., Tsao, C. Y., Horwacik, I., Kolinski, A., Rokita, H., Brecher, M., Wang, X., Ferrone, S. and Kozbor,

D., DNA vaccine expressing the mimotope of GD2 ganglioside induces protective GD2 cross-reactive antibody responses. *Cancer Res* 2005. 65: 3410-3418.

58 Forster-Waldl, E., Riemer, A. B., Dehof, A. K., Neumann, D., Bramswig, K., Boltz-Nitulescu, G., Pehamberger, H., Zielinski, C. C., Scheiner, O., Pollak, A., Lode, H. and Jensen-Jarolim, E., Isolation and structural analysis of peptide mimotopes for the disialoganglioside GD2, a neuroblastoma tumor antigen. *Mol Immunol* 2005. 42: 319-325.

59 Willers, J., Lucchese, A., Kanduc, D. and Ferrone, S., Molecular mimicry of phage displayed peptides mimicking GD3 ganglioside. *Peptides* 1999. 20: 1021-1026.

60 Monzavi-Karbassi, B., Cunto-Amesty, G., Luo, P., Shamloo, S., Blaszczyk-Thurin, M. and Kieber-Emmons, T., Immunization with a carbohydrate mimicking peptide augments tumor-specific cellular responses. *Int Immunol* 2001. 13: 1361-1371.

61 Kowalczyk, A., Wierzbicki, A., Gil, M., Bambach, B., Kaneko, Y., Rokita, H., Repasky, E., Fenstermaker, R., Brecher, M., Ciesielski, M. and Kozbor, D., Induction of protective immune responses against NXS2 neuroblastoma challenge in mice by immunotherapy with GD2 mimotope vaccine and IL-15 and IL-21 gene delivery. *Cancer Immunol Immunother* 2007. 56: 1443-1458.

62 Monzavi-Karbassi, B., Luo, P., Jousheghany, F., Torres-Quinones, M., Cunto-Amesty, G., Artaud, C. and Kieber-Emmons, T., A mimic of tumor rejection antigen-associated carbohydrates mediates an antitumor cellular response. *Cancer Res* 2004. 64: 2162-2166.

63 Abdel-Motal, U. M., Berg, L., Rosen, A., Bengtsson, M., Thorpe, C. J., Kihlberg, J., Dahmen, J., Magnusson, G., Karlsson, K. A. and Jondal, M., Immunization with glycosylated Kb-binding peptides generates carbohydrate-specific, unrestricted cytotoxic T cells. *Eur J Immunol* 1996. 26: 544-551.

64 Wierzbicki, A., Gil, M., Ciesielski, M., Fenstermaker, R. A., Kaneko, Y., Rokita, H., Lau, J. T. and Kozbor, D., Immunization with a mimotope of GD2 ganglioside induces CD8+ T cells that recognize cell adhesion molecules on tumor cells. *J Immunol* 2008. 181: 6644-6653.

65 Bitton, R. J., Guthmann, M. D., Gabri, M. R., Carnero, A. J., Alonso, D. F., Fainboim, L. and Gomez, D. E., Cancer vaccines: an update with special focus on ganglioside antigens. *Oncol Rep* 2002. 9: 267-276.

66 Merzak, A., Koochekpour, S. and Pilkington, G. J., Cell surface gangliosides are involved in the control of human glioma cell invasion in vitro. *Neurosci Lett* 1994. 177: 44-46.

67 Ravindranath, M. H., Tsuchida, T., Morton, D. L. and Irie, R. F., Ganglioside GM3:GD3 ratio as an index for the management of melanoma. *Cancer* 1991. 67: 3029-3035.

68 Livingston, P., Ganglioside vaccines with emphasis on GM2. *Semin Oncol* 1998. 25: 636-645.

69 Saleh, M. N., Khazaeli, M. B., Wheeler, R. H., Allen, L., Tilden, A. B., Grizzle, W., Reisfeld, R. A., Yu, A. L., Gillies, S. D. and LoBuglio, A. F., Phase I trial of the

chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma. *Hum Antibodies Hybridomas* 1992. 3: 19-24.

70 Scott, A. M., Lee, F. T., Hopkins, W., Cebon, J. S., Wheatley, J. M., Liu, Z., Smyth, F. E., Murone, C., Sturrock, S., MacGregor, D., Hanai, N., Inoue, K., Yamasaki, M., Brechbiel, M. W., Davis, I. D., Murphy, R., Hannah, A., Lim-Joon, M., Chan, T., Chong, G., Ritter, G., Hoffman, E. W., Burgess, A. W. and Old, L. J., Specific targeting, biodistribution, and lack of immunogenicity of chimeric anti-GD3 monoclonal antibody KM871 in patients with metastatic melanoma: results of a phase I trial. *J Clin Oncol* 2001. 19: 3976-3987.

71 Minasian, L. M., Szatrowski, T. P., Rosenblum, M., Steffens, T., Morrison, M. E., Chapman, P. B., Williams, L., Nathan, C. F. and Houghton, A. N., Hemorrhagic tumor necrosis during a pilot trial of tumor necrosis factor-alpha and anti-GD3 ganglioside monoclonal antibody in patients with metastatic melanoma. *Blood* 1994. 83: 56-64.

72 Schulz, G., Cheresh, D. A., Varki, N. M., Yu, A., Staffileno, L. K. and Reisfeld, R. A., Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. *Cancer Res* 1984. 44: 5914-5920.

73 Cheresh, D. A., Varki, A. P., Varki, N. M., Stallcup, W. B., Levine, J. and Reisfeld, R. A., A monoclonal antibody recognizes an O-acylated sialic acid in a human melanoma-associated ganglioside. *J Biol Chem* 1984. 259: 7453-7459.

74 Cheung, N. K., Saarinen, U. M., Neely, J. E., Landmeier, B., Donovan, D. and Coccia, P. F., Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells. *Cancer Res* 1985. 45: 2642-2649.

75 Yu, A. L., Uttenreuther-Fischer, M. M., Huang, C. S., Tsui, C. C., Gillies, S. D., Reisfeld, R. A. and Kung, F. H., Phase I trial of a human-mouse chimeric anti-disialoganglioside monoclonal antibody ch14.18 in patients with refractory neuroblastoma and osteosarcoma. *J Clin Oncol* 1998. 16: 2169-2180.

76 Kramer, K., Gerald, W. L., Kushner, B. H., Larson, S. M., Hameed, M. and Cheung, N. K., Disialoganglioside G(D2) loss following monoclonal antibody therapy is rare in neuroblastoma. *Clin Cancer Res* 1998. 4: 2135-2139.

77 Cheresh, D. A., Harper, J. R., Schulz, G. and Reisfeld, R. A., Localization of the gangliosides GD2 and GD3 in adhesion plaques and on the surface of human melanoma cells. *Proc Natl Acad Sci U S A* 1984. 81: 5767-5771.

78 Aixinjueluo, W., Furukawa, K., Zhang, Q., Hamamura, K., Tokuda, N., Yoshida, S., Ueda, R. and Furukawa, K., Mechanisms for the apoptosis of small cell lung cancer cells induced by anti-GD2 monoclonal antibodies: roles of anoikis. *J Biol Chem* 2005. 280: 29828-29836.

79 Cheung, N. K., Lazarus, H., Miraldi, F. D., Abramowsky, C. R., Kallick, S., Saarinen, U. M., Spitzer, T., Strandjord, S. E., Coccia, P. F. and Berger, N. A., Ganglioside GD2 specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. *J Clin Oncol* 1987. 5: 1430-1440.

80 Irie, R. F. and Morton, D. L., Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. *Proc Natl Acad Sci U S A* 1986. 83: 8694-8698.

81 Saleh, M. N., Khazaeli, M. B., Wheeler, R. H., Dropcho, E., Liu, T., Urist, M., Miller, D. M., Lawson, S., Dixon, P., Russell, C. H. and et al., Phase I trial of the murine monoclonal anti-GD2 antibody 14G2a in metastatic melanoma. *Cancer Res* 1992. 52: 4342-4347.

82 Handgretinger, R., Baader, P., Dopfer, R., Klingebiel, T., Reuland, P., Treuner, J., Reisfeld, R. A. and Niethammer, D., A phase I study of neuroblastoma with the anti-ganglioside GD2 antibody 14.G2a. *Cancer Immunol Immunother* 1992. 35: 199-204.

83 Cheung, N. K., Cheung, I. Y., Canete, A., Yeh, S. J., Kushner, B., Bonilla, M. A., Heller, G. and Larson, S. M., Antibody response to murine anti-GD2 monoclonal antibodies: correlation with patient survival. *Cancer Res* 1994. 54: 2228-2233.

84 Cheung, N. K., Kushner, B., Cheung, I. Y., Kramer, K., Canete, A., Gerald, W., Bonilla, M. A., Finn, R., Yeh, S. J. and Larson, S. M., Anti-G(D2) antibody treatment of minimal residual stage 4 neuroblastoma diagnosed at more than 1 year of age. *J Clin Oncol* 1998. 16: 3053-3060.

85 Cheung, N. K., Guo, H. F., Heller, G. and Cheung, I. Y., Induction of Ab3 and Ab3' antibody was associated with long-term survival after anti-G(D2) antibody therapy of stage 4 neuroblastoma. *Clin Cancer Res* 2000. 6: 2653-2660.

86 Modak, S. and Cheung, N. K., Disialoganglioside directed immunotherapy of neuroblastoma. *Cancer Invest* 2007. 25: 67-77.

87 Ragupathi, G., Livingston, P. O., Hood, C., Gathuru, J., Krown, S. E., Chapman, P. B., Wolchok, J. D., Williams, L. J., Oldfield, R. C. and Hwu, W. J., Consistent antibody response against ganglioside GD2 induced in patients with melanoma by a GD2 lactone-keyhole limpet hemocyanin conjugate vaccine plus immunological adjuvant QS-21. *Clin Cancer Res* 2003. 9: 5214-5220.

88 Pirofski, L. A., Polysaccharides, mimotopes and vaccines for fungal and encapsulated pathogens. *Trends Microbiol* 2001. 9: 445-451.

89 Nores, G. A., Dohi, T., Taniguchi, M. and Hakomori, S., Density-dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen: requirements for tumor-associated antigen and immunogen. *J Immunol* 1987. 139: 3171-3176.

90 Foon, K. A., Lutzky, J., Baral, R. N., Yannelli, J. R., Hutchins, L., Teitelbaum, A., Kashala, O. L., Das, R., Garrison, J., Reisfeld, R. A. and Bhattacharya-Chatterjee, M., Clinical and immune responses in advanced melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. *J Clin Oncol* 2000. 18: 376-384.

91 Rosenberg, S. A., Progress in human tumour immunology and immunotherapy. *Nature* 2001. 411: 380-384.

92 Berzofsky, J. A., Terabe, M., Oh, S., Belyakov, I. M., Ahlers, J. D., Janik, J. E. and Morris, J. C., Progress on new vaccine strategies for the immunotherapy and prevention of cancer. *J Clin Invest* 2004. 113: 1515-1525.

93 Chabalgoity, J. A., Dougan, G., Mastroeni, P. and Aspinall, R. J., Live bacteria as the basis for immunotherapies against cancer. *Expert Rev Vaccines* 2002. 1: 495-505.

94 Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D. and Mulligan, R. C., Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A* 1993. 90: 3539-3543.

95 Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R. and Gilboa, E., Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med* 1990. 172: 1217-1224.

96 Asher, A. L., Mule, J. J., Kasid, A., Restifo, N. P., Salo, J. C., Reichert, C. M., Jaffe, G., Fendly, B., Kriegler, M. and Rosenberg, S. A., Murine tumor cells transduced with the gene for tumor necrosis factor-alpha. Evidence for paracrine immune effects of tumor necrosis factor against tumors. *J Immunol* 1991. 146: 3227-3234.

97 Johnson, B. D., Gershan, J. A., Natalia, N., Zujewski, H., Weber, J. J., Yan, X. and Orentas, R. J., Neuroblastoma cells transiently transfected to simultaneously express the co-stimulatory molecules CD54, CD80, CD86, and CD137L generate antitumor immunity in mice. *J Immunother* 2005. 28: 449-460.

98 Simons, J. W., Mikhak, B., Chang, J. F., DeMarzo, A. M., Carducci, M. A., Lim, M., Weber, C. E., Baccala, A. A., Goemann, M. A., Clift, S. M., Ando, D. G., Levitsky, H. I., Cohen, L. K., Sanda, M. G., Mulligan, R. C., Partin, A. W., Carter, H. B., Piantadosi, S., Marshall, F. F. and Nelson, W. G., Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res* 1999. 59: 5160-5168.

99 Maio, M., Fonsatti, E., Lamaj, E., Altomonte, M., Cattarossi, I., Santantonio, C., Melani, C., Belli, F., Arienti, F., Colombo, M. P. and Parmiani, G., Vaccination of stage IV patients with allogeneic IL-4- or IL-2-gene-transduced melanoma cells generates functional antibodies against vaccinating and autologous melanoma cells. *Cancer Immunol Immunother* 2002. 51: 9-14.

100 Antonia, S. J., Seigne, J., Diaz, J., Muro-Cacho, C., Extermann, M., Farmelo, M. J., Friberg, M., Alsarraj, M., Mahany, J. J., Pow-Sang, J., Cantor, A. and Janssen, W., Phase I trial of a B7-1 (CD80) gene modified autologous tumor cell vaccine in combination with systemic interleukin-2 in patients with metastatic renal cell carcinoma. *J Urol* 2002. 167: 1995-2000.

101 Finn, O. J., Cancer vaccines: between the idea and the reality. *Nat Rev Immunol* 2003. 3: 630-641.

102 Parmiani, G., Castelli, C., Dalerba, P., Mortarini, R., Rivoltini, L., Marincola, F. M. and Anichini, A., Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? *J Natl Cancer Inst* 2002. 94: 805-818.

103 Lee, P., Wang, F., Kuniyoshi, J., Rubio, V., Stuges, T., Groshen, S., Gee, C., Lau, R., Jeffery, G., Margolin, K., Marty, V. and Weber, J., Effects of interleukin-12 on the immune response to a multipeptide vaccine for resected metastatic melanoma. *J Clin Oncol* 2001. 19: 3836-3847.

104 Weber, J., Sondak, V. K., Scotland, R., Phillip, R., Wang, F., Rubio, V., Stuge, T. B., Groshen, S. G., Gee, C., Jeffery, G. G., Sian, S. and Lee, P. P., Granulocyte-

macrophage-colony-stimulating factor added to a multipeptide vaccine for resected Stage II melanoma. *Cancer* 2003. 97: 186-200.

105 Phan, G. Q., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., Restifo, N. P., Haworth, L. R., Seipp, C. A., Freezer, L. J., Morton, K. E., Mavroukakis, S. A., Duray, P. H., Steinberg, S. M., Allison, J. P., Davis, T. A. and Rosenberg, S. A., Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* 2003. 100: 8372-8377.

106 Mukherji, B., Chakraborty, N. G., Yamasaki, S., Okino, T., Yamase, H., Sporn, J. R., Kurtzman, S. K., Ergin, M. T., Ozols, J., Meehan, J. and et al., Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. *Proc Natl Acad Sci U S A* 1995. 92: 8078-8082.

107 Pertl, U., Wodrich, H., Ruehlmann, J. M., Gillies, S. D., Lode, H. N. and Reisfeld, R. A., Immunotherapy with a posttranscriptionally modified DNA vaccine induces complete protection against metastatic neuroblastoma. *Blood* 2003. 101: 649-654.

108 Marshall, J. L., Hoyer, R. J., Toomey, M. A., Faraguna, K., Chang, P., Richmond, E., Pedicano, J. E., Gehan, E., Peck, R. A., Arlen, P., Tsang, K. Y. and Schlom, J., Phase I study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. *J Clin Oncol* 2000. 18: 3964-3973.

109 Zhu, M. Z., Marshall, J., Cole, D., Schlom, J. and Tsang, K. Y., Specific cytolytic T-cell responses to human CEA from patients immunized with recombinant avipox-CEA vaccine. *Clin Cancer Res* 2000. 6: 24-33.

110 Mitchison, N. A., Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J Exp Med* 1955. 102: 157-177.

111 Rosenberg, S. A., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., Restifo, N. P., Haworth, L. R., Seipp, C. A., Freezer, L. J., Morton, K. E., Mavroukakis, S. A. and White, D. E., Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen. *Hum Gene Ther* 2003. 14: 709-714.

112 Dudley, M. E. and Rosenberg, S. A., Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer* 2003. 3: 666-675.

113 Wang, L. X., Huang, W. X., Graor, H., Cohen, P. A., Kim, J. A., Shu, S. and Plautz, G. E., Adoptive immunotherapy of cancer with polyclonal, 108-fold hyperexpanded, CD4+ and CD8+ T cells. *J Transl Med* 2004. 2: 41.

114 Gattinoni, L., Powell, D. J., Jr., Rosenberg, S. A. and Restifo, N. P., Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol* 2006. 6: 383-393.

115 Gattinoni, L., Klebanoff, C. A., Palmer, D. C., Wrzesinski, C., Kerstann, K., Yu, Z., Finkelstein, S. E., Theoret, M. R., Rosenberg, S. A. and Restifo, N. P., Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest* 2005. 115: 1616-1626.

116 Dudley, M. E., Wunderlich, J. R., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R. M., Marincola, F. M., Leitman, S. F., Seipp, C. A., Rogers-Freezer, L., Morton, K. E., Nahvi, A., Mavroukakis, S. A., White, D. E. and Rosenberg, S. A., A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumor antigen-specific T lymphocytes in patients with metastatic melanoma. *J Immunother* 2002. 25: 243-251.

117 Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H. and White, D. E., Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 1994. 86: 1159-1166.

118 Gattinoni, L., Finkelstein, S. E., Klebanoff, C. A., Antony, P. A., Palmer, D. C., Spiess, P. J., Hwang, L. N., Yu, Z., Wrzesinski, C., Heimann, D. M., Surh, C. D., Rosenberg, S. A. and Restifo, N. P., Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *J Exp Med* 2005. 202: 907-912.

119 Dudley, M. E., Wunderlich, J. R., Robbins, P. F., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R., Restifo, N. P., Hubicki, A. M., Robinson, M. R., Raffeld, M., Duray, P., Seipp, C. A., Rogers-Freezer, L., Morton, K. E., Mavroukakis, S. A., White, D. E. and Rosenberg, S. A., Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002. 298: 850-854.

120 Wrzesinski, C., Paulos, C. M., Gattinoni, L., Palmer, D. C., Kaiser, A., Yu, Z., Rosenberg, S. A. and Restifo, N. P., Hematopoietic stem cells promote the expansion and function of adoptively transferred antitumor CD8 T cells. *J Clin Invest* 2007. 117: 492-501.

121 Antony, P. A., Piccirillo, C. A., Akpinarli, A., Finkelstein, S. E., Speiss, P. J., Surman, D. R., Palmer, D. C., Chan, C. C., Klebanoff, C. A., Overwijk, W. W., Rosenberg, S. A. and Restifo, N. P., CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* 2005. 174: 2591-2601.

122 Antony, P. A. and Restifo, N. P., CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. *J Immunother* 2005. 28: 120-128.

123 Turk, M. J., Guevara-Patino, J. A., Rizzuto, G. A., Engelhorn, M. E., Sakaguchi, S. and Houghton, A. N., Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J Exp Med* 2004. 200: 771-782.

124 Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M., Regulatory T cells and immune tolerance. *Cell* 2008. 133: 775-787.

125 Yamaguchi, T. and Sakaguchi, S., Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol* 2006. 16: 115-123.

126 Wang, H. Y. and Wang, R. F., Regulatory T cells and cancer. *Curr Opin Immunol* 2007. 19: 217-223.

127 Woo, E. Y., Chu, C. S., Goletz, T. J., Schlienger, K., Yeh, H., Coukos, G., Rubin, S. C., Kaiser, L. R. and June, C. H., Regulatory CD4(+)CD25(+) T cells in tumors from

patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001. 61: 4766-4772.

128 Viguier, M., Lemaitre, F., Verola, O., Cho, M. S., Gorochov, G., Dubertret, L., Bachelez, H., Kourilsky, P. and Ferradini, L., Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 2004. 173: 1444-1453.

129 Wang, R. F., Peng, G. and Wang, H. Y., Regulatory T cells and Toll-like receptors in tumor immunity. *Semin Immunol* 2006. 18: 136-142.

130 Furtado, G. C., Curotto de Lafaille, M. A., Kutchukhidze, N. and Lafaille, J. J., Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 2002. 196: 851-857.

131 Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. and Rudensky, A. Y., A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 2005. 6: 1142-1151.

132 Schluns, K. S., Kieper, W. C., Jameson, S. C. and Lefrancois, L., Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 2000. 1: 426-432.

133 Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J. and Surh, C. D., Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J Exp Med* 2002. 195: 1523-1532.

134 Prlic, M., Blazar, B. R., Farrar, M. A. and Jameson, S. C., In vivo survival and homeostatic proliferation of natural killer cells. *J Exp Med* 2003. 197: 967-976.

135 Koka, R., Burkett, P. R., Chien, M., Chai, S., Chan, F., Lodolce, J. P., Boone, D. L. and Ma, A., Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. *J Exp Med* 2003. 197: 977-984.

136 Dudley, M. E., Wunderlich, J. R., Yang, J. C., Sherry, R. M., Topalian, S. L., Restifo, N. P., Royal, R. E., Kammula, U., White, D. E., Mavroukakis, S. A., Rogers, L. J., Gracia, G. J., Jones, S. A., Mangiameli, D. P., Pelletier, M. M., Gea-Banacloche, J., Robinson, M. R., Berman, D. M., Filie, A. C., Abati, A. and Rosenberg, S. A., Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 2005. 23: 2346-2357.

137 Steinman, R. M., The control of immunity and tolerance by dendritic cell. *Pathol Biol (Paris)* 2003. 51: 59-60.

138 Schuler, G., Schuler-Thurner, B. and Steinman, R. M., The use of dendritic cells in cancer immunotherapy. *Curr Opin Immunol* 2003. 15: 138-147.

139 Steinman, R. M., Hawiger, D. and Nussenzweig, M. C., Tolerogenic dendritic cells. *Annu Rev Immunol* 2003. 21: 685-711.

140 Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. and Enk, A. H., Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 2000. 192: 1213-1222.

141 Gabrilovich, D., Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol* 2004. 4: 941-952.

142 Kalinski, P., Urban, J., Narang, R., Berk, E., Wieckowski, E. and Muthuswamy, R., Dendritic cell-based therapeutic cancer vaccines: what we have and what we need. *Future Oncol* 2009. 5: 379-390.

143 Siders, W. M., Vergilis, K. L., Johnson, C., Shields, J. and Kaplan, J. M., Induction of specific antitumor immunity in the mouse with the electrofusion product of tumor cells and dendritic cells. *Mol Ther* 2003. 7: 498-505.

144 Ashley, D. M., Faiola, B., Nair, S., Hale, L. P., Bigner, D. D. and Gilboa, E., Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. *J Exp Med* 1997. 186: 1177-1182.

145 Mayordomo, J. I., Loftus, D. J., Sakamoto, H., De Cesare, C. M., Appasamy, P. M., Lotze, M. T., Storkus, W. J., Appella, E. and DeLeo, A. B., Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. *J Exp Med* 1996. 183: 1357-1365.

146 Zitvogel, L., Mayordomo, J. I., Tjandrawan, T., DeLeo, A. B., Clarke, M. R., Lotze, M. T. and Storkus, W. J., Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* 1996. 183: 87-97.

147 Chen, H. W., Lee, Y. P., Chung, Y. F., Shih, Y. C., Tsai, J. P., Tao, M. H. and Ting, C. C., Inducing long-term survival with lasting anti-tumor immunity in treating B cell lymphoma by a combined dendritic cell-based and hydrodynamic plasmid-encoding IL-12 gene therapy. *Int Immunol* 2003. 15: 427-435.

148 Hsu, F. J., Benike, C., Fagnoni, F., Liles, T. M., Czerwinski, D., Taidi, B., Engleman, E. G. and Levy, R., Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 1996. 2: 52-58.

149 Timmerman, J. M., Czerwinski, D. K., Davis, T. A., Hsu, F. J., Benike, C., Hao, Z. M., Taidi, B., Rajapaksa, R., Caspar, C. B., Okada, C. Y., van Beckhoven, A., Liles, T. M., Engleman, E. G. and Levy, R., Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood* 2002. 99: 1517-1526.

150 Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G. and Schadendorf, D., Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998. 4: 328-332.

151 Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E. and Schuler, G., Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999. 190: 1669-1678.

152 Fong, L., Hou, Y., Rivas, A., Benike, C., Yuen, A., Fisher, G. A., Davis, M. M. and Engleman, E. G., Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc Natl Acad Sci U S A* 2001. 98: 8809-8814.

153 Schadendorf, D., Ugurel, S., Schuler-Thurner, B., Nestle, F. O., Enk, A., Brocker, E. B., Grabbe, S., Rittgen, W., Edler, L., Sucker, A., Zimpfer-Rechner, C., Berger, T., Kamarashev, J., Burg, G., Jonuleit, H., Tuttenberg, A., Becker, J. C., Keikavoussi, P., Kampgen, E. and Schuler, G., Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. *Ann Oncol* 2006. 17: 563-570.

154 Muthuswamy, R., Urban, J., Lee, J. J., Reinhart, T. A., Bartlett, D. and Kalinski, P., Ability of mature dendritic cells to interact with regulatory T cells is imprinted during maturation. *Cancer Res* 2008. 68: 5972-5978.

155 Vieira, P. L., de Jong, E. C., Wierenga, E. A., Kapsenberg, M. L. and Kalinski, P., Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol* 2000. 164: 4507-4512.

156 Mailliard, R. B., Wankowicz-Kalinska, A., Cai, Q., Wesa, A., Hilkens, C. M., Kapsenberg, M. L., Kirkwood, J. M., Storkus, W. J. and Kalinski, P., alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res* 2004. 64: 5934-5937.

157 Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K. and Steinman, R. M., Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 2003. 198: 235-247.

158 Zhou, G., Drake, C. G. and Levitsky, H. I., Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. *Blood* 2006. 107: 628-636.

159 Palucka, A. K., Ueno, H., Fay, J. W. and Banchereau, J., Taming cancer by inducing immunity via dendritic cells. *Immunol Rev* 2007. 220: 129-150.

160 Cote, A. L., Usherwood, E. J. and Turk, M. J., Tumor-specific T-cell memory: clearing the regulatory T-cell hurdle. *Cancer Res* 2008. 68: 1614-1617.

161 Ma, J., Urba, W. J., Si, L., Wang, Y., Fox, B. A. and Hu, H. M., Anti-tumor T cell response and protective immunity in mice that received sublethal irradiation and immune reconstitution. *Eur J Immunol* 2003. 33: 2123-2132.

162 Ravetch, J. V. and Lanier, L. L., Immune inhibitory receptors. *Science* 2000. 290: 84-89.

163 Dhodapkar, M. V., Dhodapkar, K. M. and Palucka, A. K., Interactions of tumor cells with dendritic cells: balancing immunity and tolerance. *Cell Death Differ* 2008. 15: 39-50.

164 Ghazizadeh, S., Bolen, J. B. and Fleit, H. B., Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. *J Biol Chem* 1994. 269: 8878-8884.

165 Rankin, B. M., Yocum, S. A., Mittler, R. S. and Kiener, P. A., Stimulation of tyrosine phosphorylation and calcium mobilization by Fc gamma receptor cross-linking. Regulation by the phosphotyrosine phosphatase CD45. *J Immunol* 1993. 150: 605-616.

166 Odin, J. A., Edberg, J. C., Painter, C. J., Kimberly, R. P. and Unkeless, J. C., Regulation of phagocytosis and [Ca2+]i flux by distinct regions of an Fc receptor. *Science* 1991. 254: 1785-1788.

167 Liao, F., Shin, H. S. and Rhee, S. G., Tyrosine phosphorylation of phospholipase C-gamma 1 induced by cross-linking of the high-affinity or low-affinity Fc receptor for IgG in U937 cells. *Proc Natl Acad Sci U S A* 1992. 89: 3659-3663.

168 Bolland, S. and Ravetch, J. V., Inhibitory pathways triggered by ITIM-containing receptors. *Adv Immunol* 1999. 72: 149-177.

169 Nimmerjahn, F. and Ravetch, J. V., Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* 2008. 8: 34-47.

170 Ravetch, J. V. and Bolland, S., IgG Fc receptors. *Annu Rev Immunol* 2001. 19: 275-290.

171 Dhodapkar, K. M., Kaufman, J. L., Ehlers, M., Banerjee, D. K., Bonvini, E., Koenig, S., Steinman, R. M., Ravetch, J. V. and Dhodapkar, M. V., Selective blockade of inhibitory Fcgamma receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A* 2005. 102: 2910-2915.

172 Kalergis, A. M. and Ravetch, J. V., Inducing tumor immunity through the selective engagement of activating Fcgamma receptors on dendritic cells. *J Exp Med* 2002. 195: 1653-1659.

173 Dhodapkar, K. M., Banerjee, D., Connolly, J., Kukreja, A., Matayeva, E., Veri, M. C., Ravetch, J. V., Steinman, R. M. and Dhodapkar, M. V., Selective blockade of the inhibitory Fcgamma receptor (FcgammaRIIB) in human dendritic cells and monocytes induces a type I interferon response program. *J Exp Med* 2007. 204: 1359-1369.

174 Nimmerjahn, F. and Ravetch, J. V., Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 2005. 310: 1510-1512.

175 Li, Q. X., Liu, G. and Wong-Staal, F., Oncolytic virotherapy as a personalized cancer vaccine. *Int J Cancer* 2008. 123: 493-499.

176 Diaz, R. M., Galivo, F., Kottke, T., Wongthida, P., Qiao, J., Thompson, J., Valdes, M., Barber, G. and Vile, R. G., Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. *Cancer Res* 2007. 67: 2840-2848.

177 Smith, G. L., Murphy, B. R. and Moss, B., Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc Natl Acad Sci U S A* 1983. 80: 7155-7159.

178 Panicali, D., Davis, S. W., Weinberg, R. L. and Paoletti, E., Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. *Proc Natl Acad Sci U S A* 1983. 80: 5364-5368.

179 Guo, Z. S., Thorne, S. H. and Bartlett, D. L., Oncolytic virotherapy: molecular targets in tumor-selective replication and carrier cell-mediated delivery of oncolytic viruses. *Biochim Biophys Acta* 2008. 1785: 217-231.

180 McCart, J. A., Ward, J. M., Lee, J., Hu, Y., Alexander, H. R., Libutti, S. K., Moss, B. and Bartlett, D. L., Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Res* 2001. 61: 8751-8757.

181 Yoo, N. K., Pyo, C. W., Kim, Y., Ahn, B. Y. and Choi, S. Y., Vaccinia virus-mediated cell cycle alteration involves inactivation of tumour suppressors associated with Brf1 and TBP. *Cell Microbiol* 2008. 10: 583-592.

182 Bollum, F. J. and Potter, V. R., Incorporation of thymidine into deoxyribonucleic acid by enzymes from rat tissues. *J Biol Chem* 1958. 233: 478-482.

183 Berk, A. J. and Clayton, D. A., A genetically distinct thymidine kinase in mammalian mitochondria. Exclusive labeling of mitochondrial deoxyribonucleic acid. *J Biol Chem* 1973. 248: 2722-2729.

184 Littlefield, J. W., The periodic synthesis of thymidine kinase in mouse fibroblasts. *Biochim Biophys Acta* 1966. 114: 398-403.

185 Murphree, A. L. and Benedict, W. F., Retinoblastoma: clues to human oncogenesis. *Science* 1984. 223: 1028-1033.

186 Singhal, S., Vachani, A., Antin-Ozerkis, D., Kaiser, L. R. and Albelda, S. M., Prognostic implications of cell cycle, apoptosis, and angiogenesis biomarkers in non-small cell lung cancer: a review. *Clin Cancer Res* 2005. 11: 3974-3986.

187 Kawamoto, H., Koizumi, H. and Uchikoshi, T., Expression of the G2-M checkpoint regulators cyclin B1 and cdc2 in nonmalignant and malignant human breast lesions: immunocytochemical and quantitative image analyses. *Am J Pathol* 1997. 150: 15-23.

188 Hengstschlager, M., Knofler, M., Mullner, E. W., Ogris, E., Wintersberger, E. and Wawra, E., Different regulation of thymidine kinase during the cell cycle of normal versus DNA tumor virus-transformed cells. *J Biol Chem* 1994. 269: 13836-13842.

189 Twardzik, D. R., Brown, J. P., Ranchalis, J. E., Todaro, G. J. and Moss, B., Vaccinia virus-infected cells release a novel polypeptide functionally related to transforming and epidermal growth factors. *Proc Natl Acad Sci U S A* 1985. 82: 5300-5304.

190 Tzahar, E., Moyer, J. D., Waterman, H., Barbacci, E. G., Bao, J., Levkowitz, G., Shelly, M., Strano, S., Pinkas-Kramarski, R., Pierce, J. H., Andrews, G. C. and Yarden, Y., Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. *Embo J* 1998. 17: 5948-5963.

191 Thorne, S. H., Hwang, T. H., O'Gorman, W. E., Bartlett, D. L., Sei, S., Kanji, F., Brown, C., Werier, J., Cho, J. H., Lee, D. E., Wang, Y., Bell, J. and Kirn, D. H., Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. *J Clin Invest* 2007. 117: 3350-3358.

192 Mendelsohn, J. and Baselga, J., Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol* 2003. 21: 2787-2799.

193 Liu, T. C. and Kirn, D., Systemic efficacy with oncolytic virus therapeutics: clinical proof-of-concept and future directions. *Cancer Res* 2007. 67: 429-432.

194 Yang, Y., Huang, C. T., Huang, X. and Pardoll, D. M., Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* 2004. 5: 508-515.

195 Zhu, J., Martinez, J., Huang, X. and Yang, Y., Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN-beta. *Blood* 2007. 109: 619-625.

196 Pasare, C. and Medzhitov, R., Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 2003. 299: 1033-1036.

197 Lode, H. N., Xiang, R., Dreier, T., Varki, N. M., Gillies, S. D. and Reisfeld, R. A., Natural killer cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy. *Blood* 1998. 91: 1706-1715.

198 Mujoo, K., Kipps, T. J., Yang, H. M., Cheresh, D. A., Wargalla, U., Sander, D. J. and Reisfeld, R. A., Functional properties and effect on growth suppression of human neuroblastoma tumors by isotype switch variants of monoclonal antiganglioside GD2 antibody 14.18. *Cancer Res* 1989. 49: 2857-2861.

199 Alexander, J., del Guercio, M. F., Maewal, A., Qiao, L., Fikes, J., Chesnut, R. W., Paulson, J., Bundle, D. R., DeFrees, S. and Sette, A., Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. *J Immunol* 2000. 164: 1625-1633.

200 Ahluwalia, A., Gokulan, K., Nath, I. and Rao, D. N., Modification of delivery system enhances MHC nonrestricted immunogenicity of V3 loop region of HIV-1 gp120. *Microbiol Immunol* 1997. 41: 779-784.

201 Brinker, K. G., Garner, H. and Wright, J. R., Surfactant protein A modulates the differentiation of murine bone marrow-derived dendritic cells. *Am J Physiol Lung Cell Mol Physiol* 2003. 284: L232-241.

202 Zeng, R., Spolski, R., Finkelstein, S. E., Oh, S., Kovanen, P. E., Hinrichs, C. S., Pise-Masison, C. A., Radonovich, M. F., Brady, J. N., Restifo, N. P., Berzofsky, J. A. and Leonard, W. J., Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. *J Exp Med* 2005. 201: 139-148.

203 Bolesta, E., Kowalczyk, A., Wierzbicki, A., Eppolito, C., Kaneko, Y., Takiguchi, M., Stamatatos, L., Shrikant, P. A. and Kozbor, D., Increased level and longevity of protective immune responses induced by DNA vaccine expressing the HIV-1 env glycoprotein when combined with IL-21 and IL-15 gene delivery. *J Immunol* 2006. 177: 177-191.

204 Nimmerjahn, F. and Ravetch, J. V., Fcgamma receptors: old friends and new family members. *Immunity* 2006. 24: 19-28.

205 Trinchieri, G., Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995. 13: 251-276.

206 Banerjee, D. K., Dhodapkar, M. V., Matayeva, E., Steinman, R. M. and Dhodapkar, K. M., Expansion of FOXP3high regulatory T cells by human dendritic cells (DCs) in vitro and after injection of cytokine-matured DCs in myeloma patients. *Blood* 2006. 108: 2655-2661.

207 Neal, Z. C., Imboden, M., Rakhmilevich, A. L., Kim, K. M., Hank, J. A., Surfus, J., Dixon, J. R., Lode, H. N., Reisfeld, R. A., Gillies, S. D. and Sondel, P. M., NXS2 murine neuroblastomas express increased levels of MHC class I antigens upon recurrence following NK-dependent immunotherapy. *Cancer Immunol Immunother* 2004. 53: 41-52.

208 Hunder, N. N., Wallen, H., Cao, J., Hendricks, D. W., Reilly, J. Z., Rodmyre, R., Jungbluth, A., Gnjatic, S., Thompson, J. A. and Yee, C., Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 2008. 358: 2698-2703.

209 Kmieciak, D., Wasik, T. J., Teppler, H., Pientka, J., Hsu, S. H., Takahashi, H., Okumura, K., Kaneko, Y. and Kozbor, D., The effect of deletion of the V3 loop of gp120 on cytotoxic T cell responses and HIV gp120-mediated pathogenesis. *J Immunol* 1998. 160: 5676-5683.

210 Gao, P., Uekusa, Y., Nakajima, C., Iwasaki, M., Nakahira, M., Yang, Y. F., Ono, S., Tsujimura, T., Fujiwara, H. and Hamaoka, T., Tumor vaccination that enhances antitumor T-cell responses does not inhibit the growth of established tumors even in combination with interleukin-12 treatment: the importance of inducing intratumoral T-cell migration. *J Immunother* 2000. 23: 643-653.

211 Amigorena, S., Fc gamma receptors and cross-presentation in dendritic cells. *J Exp Med* 2002. 195: F1-3.

212 Boruchov, A. M., Heller, G., Veri, M. C., Bonvini, E., Ravetch, J. V. and Young, J. W., Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. *J Clin Invest* 2005. 115: 2914-2923.

213 Schuurhuis, D. H., Ioan-Facsinay, A., Nagelkerken, B., van Schip, J. J., Sedlik, C., Melief, C. J., Verbeek, J. S. and Ossendorp, F., Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *J Immunol* 2002. 168: 2240-2246.

214 Wenink, M. H., van den Berg, W. B., van Riel, P. L. and Radstake, T. R., Fc gamma receptor mediated modulation of dendritic cells as a potential strategy in the battle against rheumatoid arthritis. *Neth J Med* 2006. 64: 103-108.

215 Kalinski, P., Hilkens, C. M., Wierenga, E. A. and Kapsenberg, M. L., T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 1999. 20: 561-567.

216 Sporri, R. and Reis e Sousa, C., Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* 2005. 6: 163-170.

217 Figdor, C. G., de Vries, I. J., Lesterhuis, W. J. and Melief, C. J., Dendritic cell immunotherapy: mapping the way. *Nat Med* 2004. 10: 475-480.

218 Hackstein, H. and Thomson, A. W., Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol* 2004. 4: 24-34.

219 Huang, B., Pan, P. Y., Li, Q., Sato, A. I., Levy, D. E., Bromberg, J., Divino, C. M. and Chen, S. H., Gr-1+CD115+ immature myeloid suppressor cells mediate the

development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* 2006. 66: 1123-1131.

220 Liu, V. C., Wong, L. Y., Jang, T., Shah, A. H., Park, I., Yang, X., Zhang, Q., Lonning, S., Teicher, B. A. and Lee, C., Tumor evasion of the immune system by converting CD4+CD25- T cells into CD4+CD25+ T regulatory cells: role of tumor-derived TGF-beta. *J Immunol* 2007. 178: 2883-2892.

221 Sharma, S., Yang, S. C., Hillinger, S., Zhu, L. X., Huang, M., Batra, R. K., Lin, J. F., Burdick, M. D., Strieter, R. M. and Dubinett, S. M., SLC/CCL21-mediated anti-tumor responses require IFNgamma, MIG/CXCL9 and IP-10/CXCL10. *Mol Cancer* 2003. 2: 22.

222 Sharma, S., Yang, S. C., Zhu, L., Reckamp, K., Gardner, B., Baratelli, F., Huang, M., Batra, R. K. and Dubinett, S. M., Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. *Cancer Res* 2005. 65: 5211-5220.

223 Iruretagoyena, M. I., Riedel, C. A., Leiva, E. D., Gutierrez, M. A., Jacobelli, S. H. and Kalergis, A. M., Activating and inhibitory Fcgamma receptors can differentially modulate T cell-mediated autoimmunity. *Eur J Immunol* 2008. 38: 2241-2250.

224 Dhodapkar, M. V., Krasovsky, J. and Olson, K., T cells from the tumor microenvironment of patients with progressive myeloma can generate strong, tumor-specific cytolytic responses to autologous, tumor-loaded dendritic cells. *Proc Natl Acad Sci U S A* 2002. 99: 13009-13013.

225 Groh, V., Li, Y. Q., Cioca, D., Hunder, N. N., Wang, W., Riddell, S. R., Yee, C. and Spies, T., Efficient cross-priming of tumor antigen-specific T cells by dendritic cells sensitized with diverse anti-MICA opsonized tumor cells. *Proc Natl Acad Sci U S A* 2005. 102: 6461-6466.

226 Munz, C., Steinman, R. M. and Fujii, S., Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med* 2005. 202: 203-207.