Towards immunotherapy in pediatric cancer patients

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Towards immunotherapy in pediatric cancer patients

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Voor Emma

Grijp ernaar met je natte kleine vingers
Sabbel op de kaft
Markeer de bladzijden
Kleur de figuren
Zet je eettafel ermee recht
Lach om de achterhaalde technieken
Weerleg de inhoud
Je bent en blijft mijn meest geslaagde experiment

Contents

Chapter 1

General introduction

Modified from:

Ex-vivo-generated dendritric cells for clinical trials versus in-vivo targeting.
Chapter in: The biology of dendritic cells and HIV infection.
Joannes F.M. Jacobs, Candida F. Pereira, Paul J. Tacken, I. Jolanda M. de Vries,
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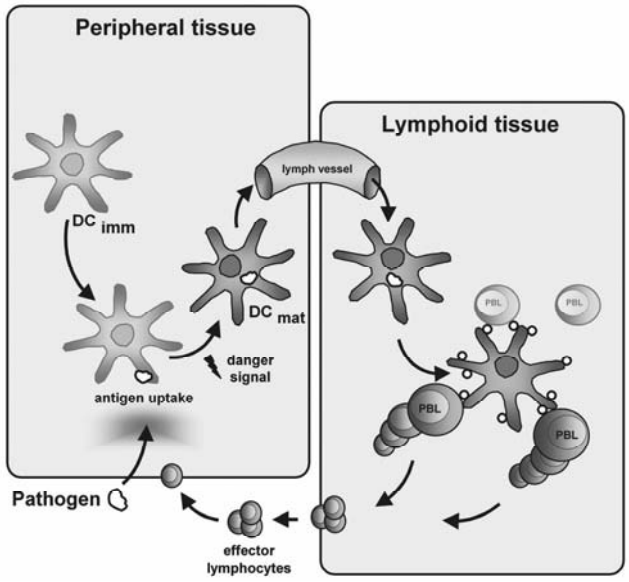
The immune system

The main function of the human immune system is to protect the body against harmful pathogens such as bacteria, parasites and viruses. The immune system is based on two distinct responses, the innate and the adaptive immune response. The innate immune response forms the first line of defense against invaded pathogens. The second line of defense is formed by the adaptive immune response. This immune response involves pathogen-specific lymphocytes, directed against selected proteins called antigens, and leads to pathogen-specific memory. The adaptive immune response can be subdivided in a cellular and humoral immune response. A cellular immune response is initiated by an antigen presenting cell that recognizes and takes up pathogens, processes antigens into small peptides that are presented via molecules of the major histocompatibility complex (MHC) to T cells. B cells have membrane immuno-globulins to bind specific antigens from the pathogen. Upon binding they convert to anti-body producing plasma cells, the so-called humoral immune response.

Figure 1



Dendritic cell life-cycle. Immature DCs reside in peripheral tissues sampling the environment. When an immature DC encounters and takes up a pathogen in the context of danger signals, they mature and migrate towards lymphoid tissues. The mature DC presents the antigens to lymphocytes. Activated effector lymphocytes subsequently invade the infected tissue and eliminate the pathogen.

Tumor immunology

For many years, it was believed that the immune system was effective only in combating infectious diseases caused by invading pathogens such as bacteria and viruses. In the 19th century, Dr. William Coley from the New York Memorial Hospital closely studied a patient who presented with recurrent sarcoma of the cheek. The extensive wound after an incomplete surgery could not be closed and skin grafts were unsuccessful. The wound became severely infected with erysipelas (Streptococcus pyogenes) and the patient developed high fevers. Surprisingly, after each attack of fever the tumor shrank, and finally disappeared completely. Dr. Coley associated the tumor regression, in this and other patients with sarcomas, with preceding infections and thereby recognized the potential role of the immune system in cancer treatment. He was the first to exploit the power of the immune system to fight cancer. He began to treat patients by injecting pre-treated bacteria, so called 'Coley vaccines', directly into inoperable tumors. Indeed, complete tumor regression was achieved in some patients after they developed a full-blown infection.[1]

The modern era of tumor immunology began several decades later with evidence that carcinogen-induced tumors harbored tumor-rejection antigens and that mice could be immunized against these transplantable tumors.[2,3] Based on these data, Thomas and Burnet pioneered the famous concept of 'immune-surveillance of cancer', to describe a mechanism that protects immune-competent hosts against cancer.[4-6]

Initial enthusiasm was tempered as the results of the first clinical trials were disappointing and it seemed that most naturally occurring tumors were 'non-immunogenic'.[7] Van Pel and Boon created a turning point by showing it is possible to induce protective immunity against so-called 'non-immunogenic' tumors, provided that the tumor cells are mutagenized.[8] Others confirmed these observations and therapeutic models were created in which immunization leads to the rejection of pre-existing tumors. The new paradigm is that the primary reason for the lack of tumor immunogenicity is the inability of the growing tumor to activate the immune system, not the absence of tumor-rejection antigens. This notion intensified the quest for ways to initiate de novo immune responses against tumors, thereby aiming at the specific eradication of cancer cells, whilst leaving normal tissues untouched.[9]

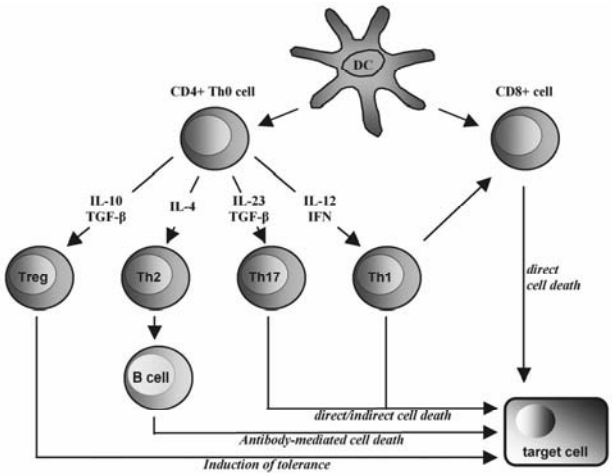
Dendritic cells: a potent tool for cancer immunotherapy

The regulation of immunity and tolerance is not only determined by T cell receptor specificity, but also by the context in which the antigens are presented to the immune system. Antigen presentation is orchestrated by professional antigen presenting cells called dendritic cells (DCs). They play a crucial role in both initiation and modulation of the immune response. DCs have the unique ability to take up and process antigens in the peripheral blood and tissues. In the presence of inflammation or endogenous danger-signals, the immature DCs undergo a maturation process. Mature DCs subsequently migrate to draining lymph nodes, where they present antigen to resting lymphocytes (figure 1).[10] Depending on the activation state of DCs, they can either be immunostimulatory or tolerogenic. Immunostimulatory DCs stimulate immune responses by activating T and B cells. Tolerogenic DCs inhibit immune responses by

induction of anergic T cells and the activation of a specific T cell subset with immune-regulating capacities, the regulatory T cells (figure 2).[11]

The lack of immunogenicity of naturally occurring tumors can now be understood in terms of suboptimal danger signals in the tumor microenvironment to generate protective immunity, and not due to the lack of tumor antigens. The tumor microenvironment does not provide adequate signals for DC activation and migration, resulting in a weak and ineffective immune response. The purpose of DC-based specific active immunotherapy is to stimulate anti-tumor immune responses by pulsing tumor antigens onto the appropriate DC subset, provide optimal conditions for the maturation of DCs, and thereby induce in-vivo specific T cell responses against the tumor. Our increased understanding of DC biology and the possibility to obtain large numbers of DCs *in vitro* from isolated monocytes has boosted the use of DCs in tumor immunotherapy.[12,13]

Figure 2



Induction of T cell responses following interaction with a dendritic cell. Under the influence of specific cytokines, progenitor T cells (Th0) differentiate into functionally distinct T cell subsets. The balance of $CD8^+$, Th1, Th2, Th17 and Treg cells directs the immune response towards pathogen destruction, autoimmunity or tolerance.

Dendritic cell culture: from bench to clinical grade product

Introduction

Since DCs mainly reside in the peripheral tissues and the lymphoid organs, only a small amount of these cells can be isolated from the peripheral blood.[14] To overcome this problem, DCs can be generated from their blood precursors. DCs originate from $CD34^+$ bone marrow stem cells differentiating into either myeloid or lymphoid precursors. These precursors subsequently differentiate into Langerhans cells, interstitial DCs or plasmacytoid DCs.[15] All subtypes have their own phenotype and specialized function.

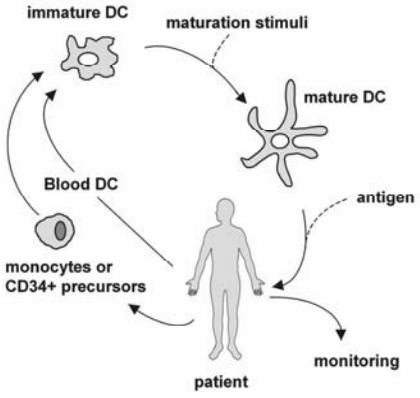
Figure 3 demonstrates a DC-vaccination protocol. These protocols are mostly used to treat cancer-patients.[16] However, new insights have broaden the application of DCs for the treatment of autoimmune diseases, allergy, therapy-resistant infections such as HIV infection and prevention of transplant rejection.[17-20] Each DC vaccination study must be carefully designed to fit its specific purpose. There are four important issues to consider in designing effective cancer vaccines: how to identify potent tumor antigens; how to stimulate an effective anti-tumor immune response; how to avoid auto-immune pathology and how to prevent immune evasion.[21] In addition, for clinical application, the DC vaccine must be developed in a GMP-compatible and reproducible procedure. In the following paragraphs, the basic principles of DC culture from monocyte precursors are outlined as they are used most widely. However, clinical DC vaccination studies have also been conducted with DCs derived from $CD34^+$ progenitor cells and DCs directly isolated from peripheral blood.[22]

Precursor isolation and differentiation into DC phenotype

It is relatively easy to isolate large numbers of monocytes for clinical use. They can be purified from peripheral blood by a variety of methods, including immuno-selection based on CD14 expression, counterflow elutriation based on specific size and weight, and adherence based on the capacity of monocytes to adhere to plastic. In general, sufficient DCs for a vaccination trial can be cultured from 500 ml peripheral blood.[23] However, additional DCs are needed for multiple vaccinations, monitoring purposes and different routes of administration. Therefore, autologous DCs for vaccination trials are usually cultured from leukapheresis products.

In 1994, it was discovered that GM-CSF and IL-4 promote differentiation of monocytes into immature DCs over a period of three to five days.[24,25] This allowed the generation of large numbers of DCs and boosted understanding of DC biology. Pioneering clinical studies with DCs quickly followed, initially focusing on immunotherapy against various cancers. New insights have broadened the application of DCs for the treatment of autoimmune diseases, the prevention of transplant rejection and therapy-resistant infections such as HIV infection.[26,27]

Figure 3



Schematic representation of DC-vaccination treatment protocol. DCs cultured directly from monocytes, $CD34^+$ progenitor cells, or directly isolated from blood can be loaded ex vivo with antigen, cultured in the presence of maturation stimuli such as proinflammatory cytokines and subsequently administered to patients.

Maturation

Immature DCs have the unique ability to take up and process antigens in peripheral tissues. *In vivo*, DC maturation occurs in response to microenvironmental signals that allow DCs to switch their functional phenotype. In the presence of 'danger' signals, DCs can undergo this maturation process.[28] Danger signals can be generated by tissue damage, inflammatory mediators, or directly by microbial products such as lipopolysaccharide and peptidoglycans associated with bacterial cell walls. The maturation process can be mimicked *in vitro* by a one- to two-day culture protocol using various exogenous stimuli, such as LPS, dsRNA, apoptotic cells, immune complexes, CpG DNA, proinflammatory cytokines and prostaglandins. Jonuleit and his colleagues first standardized the maturation procedure under GMP conditions. Their efforts led to the current 'gold standard' method of DC maturation with a cocktail of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6 and prostaglandin E_2), now known as the 'Jonuleit cocktail'.[29]

During maturation, DCs lose their ability to efficiently take up and process antigens and, instead, acquire the capacity to migrate toward lymphoid organs. In the T-cell rich parafollicular areas of lymph nodes, several chemokines, such as CCL19 and CCL21, are produced.[30] Mature DCs respond to these chemokines as a consequence of up-regulation of chemokine receptors such as CCR7.[31,32] After arrival of the mature DC in the lymph node, its main function is to activate lymphocytes. In order to achieve this, the mature DCs express antigenic peptides on their MHC complexes, upregulate expression of co-stimulatory molecules and secrete cytokines. The principal co-stimulatory molecules CD80 and/or CD86, intracellular adhesion molecule 1 (ICAM-1) and/or ICAM-2, and OX40 ligand interact with their respective T-cell counter-receptors, CD28, LFA-1 and OX40. The activation signals received by a DC determine its activation program, including which cytokines are produced. These cytokines determine the type of immune response that is induced. Although the exact nature of the signals that induce maturation are not completely understood, there is evidence that DCs matured with TNF-α and IL-1β mainly result in CTL-polarizing DCs and DCs matured with IFN-γ results in Th1-polarizing DCs.[33] Other studies report that the most effective maturation signals must come from microbial and viral products themselves (pathogen-associated molecular patterns). They are directly recognized by pattern-recognition receptors such as members of the Toll-like receptor family. Pattern-recognition receptors control the expression of genes that directly signal for DC maturation.[34]

The immune target

Both $CD8^+$ T cells and $CD4^+$ T cells recognize antigens presented as small peptides in the groove of human leukocyte antigen (HLA; the human analogue of the major histocompatibility complex). $CD8^+$ cells recognize small peptides derived from intracellular cytoplasmic proteins, digested in proteasomes and presented on cell surface class I molecules. In contrast, $CD4^+$ cells present engulfed extracellular proteins, digested to peptides in intracellular endosomes and presented on cell-surface class II HLA molecules. The antigen source and the method of antigen loading can direct an immune response towards a $CD8^+$, $CD4^+$ or combined immune response.

MHC class I and class II loading

The major route for presentation of exogenous antigens that enter the DC via endocytosis or phagocytosis is via MHC class II. Following uptake, soluble and particulate antigens are directed to the MHC class II compartments (MIIC), where they are degraded into peptide fragments and loaded onto MHC class II molecules. In general, strategies involving pulsing DCs with protein antigens or whole targets will induce class II presentation.

The class I pathway is mainly involved in presentation of endogenous self- and viral antigens that are present in the cytosol. These cytosolic proteins are degraded into peptide fragments by the proteasome. Subsequently, the peptides are transported into the endoplasmatic reticulum (ER) by Transporters associated with Antigen Processing (TAP), where they are loaded onto MHC class I molecules. This implies that strategies delivering antigens directly into the cytosol of DCs, such as loading DCs with RNA or DNA encoding antigens, will efficiently induce class I presentation. In addition, DCs have the capacity to present exogenous antigens on MHC class I via the process of cross-presentation. This process provides internalized proteins access to the cytosolic proteasome and their derived peptides access to the ER-based class I processing machinery [35]. Particulate antigens taken up by phagocytosis have access to this machinery since phagosomes fuse with the ER soon after or during their formation.[36] Soluble antigens are less efficiently cross-presented than particulate antigens.[37,38] However, internalized soluble proteins can escape proteolysis and gain access to the lumen of the ER, from where they might be transported to the cytosol and processed by the proteasome.[39]

Antigen source

The goal of anti-tumor DC-based vaccines is to induce strong immune responses against a tumor associated antigen. The choice of antigen source and loading method is a crucial step for the development of DC-based vaccines that 1) induce both humoral and cellular immune responses; 2) target a wide range of antigens; 3) avoid unwanted autoimmune responses; 4) are applicable to patients with different HLA types and 5) meet the safety requirements.

Loading DCs with peptides. Peptides derived from pathogens and tumors have been extensively used for loading of DCs to induce an immune response against those targets. The main advantage of using a defined peptide is to generate an immune response that is very specific for that epitope. This approach minimizes the risk of autoimmunity and unwanted tissue damage. Peptide vaccination is often associated with epitope spreading, which is the generation of an immune response against distinct antigens released from the target cells eliminated by the vaccine.[40] Furthermore, loading DCs with peptides circumvents the use of dangerous pathogens or infected cells and facilitates quality control. In addition, the immune response can be accurately monitored since the immune target is well defined. These properties render peptides very attractive for clinical vaccination trials.

However, the use of peptides has several disadvantages that may limit the capacity for clinical application. First, the target epitope must be defined. The use of peptides is therefore limited to those targets for which rejection antigens are

identified. Second, the induction of a $CD4^+$ and $CD8^+$ mediated immune response requires a combination of both MHC class I and class II peptides.[41] Third, because of the MHC restriction of the peptides, they are only applicable to MHC-matched patients. Therefore, large clinical studies using peptides are usually restricted to common HLA-types such as HLA-A1 or HLA-A2. The need for MHC typing and peptide identification can be circumvented by using acid-eluted peptides from autologous tumor cells, however the large amount of fresh tumor tissue needed for this procedure is often not available. Moreover, tumor cells also present shared self antigens, which could give rise to unwanted autoimmune responses.[42] Fourth, a single mutation can cause pathogen escape from immune recognition.[43] The selection of a broader spectrum of peptides may prevent the escape mechanism by loss of epitope variants.

Loading DCs with proteins. When a pathogenic antigen has been defined, but the peptide epitope has not yet been identified, the DC loading with the whole protein is a possibility. This does not only circumvent the need for identifying peptide epitopes, but also expands the clinical application to patients who are excluded due to the MHC-restriction associated with peptide loading. Since proteins are only efficiently taken up by immature DC; protein loading should occur at an immature state after which maturation can be induced.[44]

Loading DCs with whole tumor. When immunogenic antigens have not yet been identified for a certain target or a broad T cell immune response is needed, DCs can be loaded with tumor lysate. This may reduce the possibility of target escape by loss of epitope variants, as discussed above. Lysates of necrotic and apoptotic tumor cells can be made by mechanical/thermal lysis and UV irradiation, respectively. In-vivo, it is hypothesized that necrotic cells induce immunity and apoptotic cells induce tolerance. The in-vitro relevance of this is questionable since the DCs can be exposed to maturation stimuli after loading with apoptotic tumor cells.[45,46] A concern relevant to the use of whole tumor cell-derived material is the risk of transferring immunosuppressive factors from the tumor cells to the DC. If the target is processed and presented through both MHC class I and II, a more potent immune response is generated because $CD4^+$ Th cells play a critical role in inducing and maintaining effective CTL responses.[47] Especially for tumor therapy, the main disadvantage of using whole target is that the generated immune response could be less specific and may cause more concern for autoimmune diseases. Specific immune monitoring is also more difficult since the epitopes involved are not known. An additional disadvantage is that a relatively high amount of sterile tumor cells is required.

Loading DCs with RNA or DNA. Using RNA isolated from tumor cells to deliver tumor antigens to DCs has some unique advantages. First, sufficient RNA can be generated from a small amount of tissue by amplification of the RNA. Second, target restricted RNA can be enriched before DC loading by subtractive hybridization with RNA from normal tissues. Tumor-specific immune responses are thereby augmented and the risk of autoimmunity is reduced. Major drawback for the use of RNA is the instability of RNA products and the greater technical demands.[48] In addition to RNA, the use of DNA that encodes for target antigen has the advantage of expressing the antigen within the cell. Therefore, the antigen can be processed and presented in a way that closely resembles the processing of endogenous proteins. Because DNA is

less prone to degradation than RNA, antigen presentation may last longer. However, the generation of immunocompetent DCs with target DNA remains difficult because of the limitations of the DNA delivery systems and the safety concerns that are raised as the DNA integrates into the host genome.[49,50]

Methods for loading dendritic cells with antigen

Although several methods for loading DCs with antigen have been described,[51] the loading method is often restricted by the choice of antigen. Synthetic peptides are effective when pulsed onto mature DCs. In contrast, proteins and whole targets are usually loaded into immature DCs. If a whole target is used, immature DCs can be incubated with apoptotic cells, necrotic cells, cell lysates or inactivated pathogens. Afterwards, the immature DCs must be fully matured before administration into patients. The most common method for introducing RNA into the DC is electroporation.[52] Genetically modified recombinant vectors are known as the most suitable vehicle for DNA delivery into DCs (gene therapy). Recombinant vector vaccines use attenuated virus or bacteria as carriers of modified target antigens into the host. The target genetic information is incorporated into the vector genome and target proteins are produced.

A more experimental method is targeting antigen to specific receptors on the DC. Several receptors expressed on the surface of DCs are capable of delivering antigens into the DC for MHC-mediated processing and presentation. They include Fc gamma-receptors, receptors for mannose, heat shock proteins, C-type lectins and DEC-205. Current trials focus on the use of these receptors for *in-vivo* targeting of DCs.[53]

Storage

Culturing DCs is a time-consuming process. To prevent multiple culture steps, the effect of freezing DCs has been investigated.[54,55] It is demonstrated that DCs can be stored for long time periods in liquid nitrogen. Both phenotype and function are unaffected; however, up to 20% of the DCs can be lost due to the freezing procedure. Based on these results, it is common practice to culture all DCs in one procedure, divide them into lots and freeze the DCs for administration at later time points.

Dendritic cell quality check

Introduction

Poor quality vaccines are not only a health hazard, but they also bias research results and are therefore a waste of research funds. Good manufacturing practice (GMP) is a system to ensure that products are consistently produced and controlled according to quality standards. It is designed to minimize the risks involved in any pharmaceutical production process. WHO has established detailed guidelines for good manufacturing practice; these are available online. Before administration to patients, microbiological tests must rule out the possibility of any bacterial or fungal contamination.

A prerequisite for DCs used in clinical vaccination studies is that they are cultured under GMP conditions; however, it is also important that the DCs retain their functional properties. Therefore, *ex-vivo* generated DCs for clinical use must meet several quality criteria. Morphological and functional criteria, and guidelines for DC quality controls have been published.[16,56]

Phenotype and purity
Flow cytometry with antibodies directed against cell surface markers demonstrates whether all necessary molecules are expressed. *Receptors for antigen recognition and uptake:* Fc receptors (CD32 and CD64), complement receptor C3bi (CD11b), which increase efficiency of immune complex endocytosis. C-type lectin receptors, such as the macrophage mannose receptor, DEC-205 and DC-SIGN, which bind pathogenic carbohydrates. *Antigen presenting molecules:* CD1a, MHC-I and MHC-II. *Accessory/costimulatory molecules:* LFA-3 (CD59), B7-1 (CD80), B7-2 (CD86), CD40 and ICAM-1 (CD54). Monoclonal antibodies against DC specific markers can measure the purity of the DC product. The presence of low numbers of contaminating lymphocytes and monocytes in a final batch is common but not desirable.

Function
Proper functioning of DCs is evaluated by measuring cytokine release using multiplex technologies. IL-12 production has always been of specific interest as it is considered the most important cytokine for CTL and Th1 induction. The IL-12 production can be significantly impaired if monocytes are even transiently exposed to endotoxin during culture.[57] To test the proper functioning of DCs, several assays are available which mainly focus on their ability to induce T cell proliferation (for example in a mixed lymphocyte reaction) and their ability to migrate using *in-vitro* DC-migration models.[58]

Vaccine administration

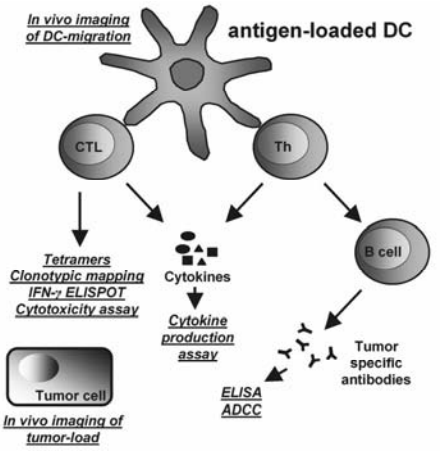
The ability of DCs to migrate to appropriate regions of lymphatic tissues is critical for the success of DC-based vaccines. DCs administered intravenously or intradermally can end up in the spleen and draining lymph nodes, respectively. However, using radiolabeled DCs demonstrated that only small percentages reach these lymphoid organs.[58,59] Some clinical trials try to overcome this by directly injecting DCs into lymph vessels or, under ultrasound guidance, into lymph nodes. Recent data suggest that the location of the primary immune response can determine the distribution of effector cells at different sites of the body.[60] DCs injected intravenously home to the spleen and induce visceral immunity. DCs administered subcutaneously or directly in peripheral lymph nodes lead to the control of cutaneous lesions. Animal experiments have not provided a clear-cut set of guidelines for DC administration; therefore, the different routes are often combined.

Immuno-monitoring

Although clinical efficacy as demonstrated by tumor regression and increased patient survival are the ultimate goals of DC vaccines, monitoring of the immunologic outcome facilitates the interpretation of study results. Immuno-monitoring methods depend mainly on the vaccine design (figure 4). In general, enhancement of the frequency of antigen-specific lymphocytes in the peripheral blood is considered as an important end-point of the patient's response to the injected vaccine. Tumor specific $CD4^+$ and $CD8^+$ T cells circulating in peripheral blood in response to the DC vaccination can be quantified with IFN-γ release measured in an ELISPOT assay and with tetramer analysis to defined epitopes. For insight in humoral immune responses, tumor specific antibody titers can be determined by ELISA.[61]

Specific T cell functionality can be determined by intracellular cytokine production or in a cytotoxicity assay such as the chromium release assay. The delayed type hypersensitivity assay is another functional monitoring assay in which antigen, alone or loaded on DCs, is injected intradermally. If functional specific T cells are present, a local immune response can be observed. The exact nature of this immune response can be analyzed by immunohistochemistry and by analyzing freshly isolated lymphocytes from the lesion.[62]

Figure 4



Monitoring tools. Several assays have been developed to determine the phenotypical and functional characteristics of the cellular and humoral immune responses. The combined use of these assays with clinical responses provides information about the effectiveness of the immunotherapeutic trial. ADCC= antibody dependent cell-mediated cytotoxicity.

The tumor microenvironment

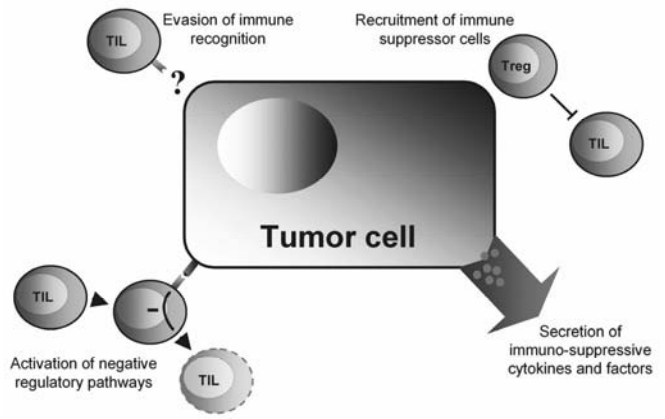
Accumulating evidence indicates that a dynamic interplay between tumors and the immune system can regulate tumor growth and metastasis.[63] The host's immune system not only protects against tumor development, but can also inadvertently promote tumor growth by selecting tumor-variants with reduced immunogenicity. Tumor immune-escape can be based on four mechanisms: evasion of immune-recognition, activation of negative co-stimulatory signals, secretion of immuno-suppressive factors and recruitment of immune-suppressor cells (figure 5).

Immune evasion. To elude immune recognition, tumor cells employ several mechanisms. Tumors can down-regulate antigen expression,[64] or accumulation of point mutations can result in cell surface antigens that can no longer be recognized by specific T cells.[65] An other commonly used mechanism by tumors to compromise immune recognition is down-regulation or shielding of MHC cell surface molecules,[66] or interference with other components of the antigen presentation machinery including the antigen transport machinery and the proteasome.[67,68]

Activation of negative costimulatory signals. Several studies indicate that molecules that convey negative regulatory signals towards effector T cells are expressed on tumor cells. FasL expressed on tumor cells can deliver death signals to Fas-positive tumor infiltrating effector T cells.[69,70] Programmed death receptor ligand-1 (PD-L1) expression on tumor cells interacts with the receptor PD-1 expressed on activated tumor infiltrating lymphocytes and interferes with T cell proliferation, cytokine secretion and induces apoptosis.[71,72] In a addition, certain sugar-structures on the tumor cell surface such as galectins and gangliosides, have the potential to induce T cell apoptosis and regulate effector functions.[73,74]

Secretion of immuno-suppressive factors. Tumors can secrete a variety of cytokines or soluble factors, such as TGF-β, IL-10, prostaglandin E2 and indoleamine 2,3 dioxygenase (IDO), that create an immune-suppressive tumor microenvironment. All these mediators have in common that they either directly, or indirectly, compromise T cell function or survival.[75,76]

Figure 5



Immunosuppressive strategies used by tumors to evade immune responses. Tumor immune-escape can be based on four general mechanisms: evasion of immune-recognition, activation of negative co-stimulatory signals, secretion of immuno-suppressive factors and recruitment of immune-suppressor cells.

Recruitment of immune-suppressive cells. Tregs contribute to prevention of autoimmune disorders by regulating function and proliferation of T lymphocytes.[77] Through the secretion of selected chemokines, cancer cells can stimulate recruitment, differentiation and/or expansion of regulatory T cells.[78] Recent studies have demonstrated that immunosuppression mediated by Tregs is one of the most critical mechanisms of tumor immune-escape.[79] In addition, tumor cells can recruit tolerogenic dendritic cells that can induce antigen-specific tolerance[80] and myeloid suppressor cells that induce T cell inhibition by secretion of reactive oxygen species.[81]

Tumor-immune escape mechanisms may have a dramatic effect on immune-therapeutic interventions. A better understanding of the interactions between tumors and the immune system will lead to novel and more effective cancer immunotherapy strategies. It will be of critical importance to determine the effectiveness of combined strategies involving immunotherapy together with blockade of immune-inhibition in the tumor microenvironment.[82,83] In fact, progress has been made in this field by evaluating the effects of anti-tumor immunotherapeutic intervention combined with CTLA-4 blockade or Treg depletion.[84,85]

Scope of this thesis

Dendritic cells are the most potent antigen-presenting cells of the immune system and represent a promising tool in the therapeutic vaccination against cancer. The scope of this thesis was to exploit the possibilities of dendritic cell-based immunotherapy in pediatric patients with high-risk solid tumors.

In **chapter 2** we investigated the feasibility of generating ex vivo clinically grade, monocyte derived DCs from pediatric cancer patients. In **chapter 3** we discuss the current status of cancer immunotherapy in children. Most immunotherapeutic therapies are still experimental and focus on adult patients. However, the first immunotherapeutic trials for pediatric cancer patients have been published and more are ongoing. To facilitate future active immunotherapy we provide an up to date overview of tumor-specific antigens expressed on pediatric solid tumors that are recognized by T cells.

The expression pattern of shared tumor-specific antigens is studied in a wide variety of adult malignancies. Some of these shared tumor-specific antigens, such as the antigens encoded by cancer-germline genes, are already widely used as immune target in clinical trials. For potential use of these antigens in pediatric oncology, we analysed cancer-germline gene expression in a large panel of pediatric solid extra-cranial tumors in **chapter 4** and in **chapter 5** we studied their expression in pediatric brain tumors.

Cellular immunotherapy is an appealing approach to treat a child because of its mild toxicity. However, we experienced that DC vaccinations in adults can induce autoimmunity. In **chapter 6** we show that melanoma patients can develop vitiligo after immunotherapy using DCs loaded with melanocyte differentiation antigens. In this chapter we describe the immunological and clinical relevance of the vaccine-induced

vitiligo. Based on our experience with DC vaccinations in melanoma patients and our pre-clinical data regarding DC culture in pediatric patients and antigen expression on pediatric solid tumors, we introduced DC-immunotherapy for pediatric patients with high risk solid tumors. In **chapter 7** we describe a clinical feasibility trial in which pediatric patients with high risk solid tumors are vaccinated with mature, monocyte-derived DCs loaded with tumor specific peptides. In this chapter we further describe the inclusion, treatment and follow-up of the first included pediatric patient.

Pre-clinical and clinical data show that cancer cells can acquire properties that allow them to evade immune attack. One 'tumor immune-escape' mechanism is the recruitment of regulatory T cells (Tregs). In **chapter 8** we provide an overview of the different suppressive mechanisms employed by Tregs. In **chapter 9** we investigated the role of Tregs in a variety of human brain tumors. To study immune cells in the brain tumor microenvironment, we developed a new method to isolate immune cells from ultrasonically resected brain tumor material.

Finally, in **chapter 10** we discuss the results, the significance and the future implications of the novel findings described in this thesis.

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Chapter 2

Phenotypic and functional characterization of mature dendritic cells from pediatric cancer patients

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Abstract

Background: Dendritic cells (DCs) are the most potent antigen presenting cells of the immune system. Clinical trials have demonstrated that mature DCs loaded with tumor associated antigens can induce tumor specific immune responses. Theoretically, pediatric patients are excellent candidates for immunotherapy since their immune system is more potent compared to adults. We studied whether sufficient amounts of mature monocyte derived DCs can be cultured from peripheral blood of pediatric cancer patients. *Procedure:* DCs from fifteen pediatric patients with an untreated primary tumor were cultured from monocytes and matured with clinical grade cytokines. Phenotype and function were tested with flow cytometry, mixed lymphocyte reaction and an *in-vitro* migration assay. DCs of children with a solid tumor were compared with monocyte derived DCs from age-related non-malignant controls. *Results:* E*x-vivo* generated monocyte derived DCs from pediatric patients can be generated in numbers sufficient for DC vaccination trials. Upon cytokine stimulation the DCs highly up-regulate expression of the maturation markers CD80, CD83 and CD86. The mature DCs are six times more potent in inducing T cell proliferation compared to immature DCs. Furthermore, mature DCs, but not immature DCs, express the chemokine receptor CCR7 and have the capacity to migrate *in-vitro*. *Conclusions:* All together these data indicate that mature DCs can be generated *ex-vivo* to further optimize DC-vaccination trials in pediatric cancer patients.

Introduction

Although major advances in the treatment of childhood malignancies have been made, cancer remains the second most common cause of death for children > 1 year of age in the developed world.[1] Beside that, most cancer therapies are associated with significant toxicity leading to long-term morbidity and an increased second malignancy rate.[2,3] Therefore, new treatment strategies against pediatric malignancies, such as immunotherapy, are warranted.[4]

Dendritic cells (DCs) are antigen-presenting cells with the unique ability to take up and process antigens in the peripheral blood and tissues. They subsequently migrate towards draining lymph nodes, where they present antigen to resting lymphocytes.[5] The understanding of DC biology and the recent progress in obtaining large numbers of *ex-vivo* generated DCs from isolated monocytes has boosted the use of DCs in tumor immunotherapy.[6] Several murine and human studies demonstrate that it is possible to induce specific anti-tumor immune responses and even tumor regression by injecting DCs pulsed with tumor lysates or tumor-specific peptides.[7-10] The immunizing ability of DCs *in-vivo* is critically influenced by their maturation state and their capacity to migrate towards lymphatic tissue. Recent reports show that compared to immature DCs, mature DCs have a higher potency to induce specific immune responses and to migrate both *in-vitro* and *in-vivo.*[11-15]

Theoretically, pediatric patients are excellent candidates for immunotherapy since their immune system is more potent compared to adults.[16,17] Two phase I/II studies have described the use of immature DCs in anti-tumor vaccines in pediatric cancer patients. These two studies reported promising results with both immunological and clinical responses.[18,19] Vaccine efficacy could be further optimized by using mature DCs instead of immature DCs since it is shown that maturation is a prerequisite for inducing immune responses in adult cancer patients.[11,15] This hypothesis is supported by a DC-based tumor vaccination study against malignant glioma in which 4 patients were included with ages ranging from 11 to 17 years. A clinical effect was seen in two of these patients.[20,21] However, the efficiency of the *ex-vivo* generation of mature DCs from young cancer patients might be impaired because several studies demonstrate that DCs can be deficient or non-functional *in-vivo* in pediatric patients with certain malignancies.[22-24]

In this study we investigate whether it is possible to generate sufficient amounts of functional mature DCs from cancer patients in the age-range of 1 to 16 years.

Methods

Patient selection-criteria

Inclusion criteria were: histological evidence of newly diagnosed, untreated malignant extra-cranial tumor. Patients must be under 18 years old. Blood samples were obtained after informed consent from age-appropriate patients and their caretaker. Results are compared with DCs isolated from age-related hospital-controls (controls) with non-malignant hematological disorders (n=5) and patients undergoing minor surgery (n=10). The study was approved by the Institutional Review Board.

Antibodies and immunostaining

The following monoclonal antibodies (mAbs) were used for flow cytometry (Beckton and Dickinson & Co., Oxnard, CA): anti-HLA ABC (W6/32), anti-HLA DR/DP (Q5/13), anti-CCR7 (kindly provided by Dr. Martin Lipp, Berlin, Germany), anti-CD80 (Beckton Dickinson, Mountain View, CA), anti-CD83 (Beckman Coulter, Mijdrecht, the Netherlands), anti-CD86 (BD Pharmingen, San Diego, CA).

DC preparation

DCs were generated as described previously.[11,25] PBMCs were isolated from 10 ml heparinized blood by Ficoll (1.077) density gradient centrifugation (25 min, 20ºC, 2100 RPM; Pharmacia). Plastic adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, Maryland) supplemented with 2% pooled human serum (PAA laboratories, Linz, Austria), IL-4 (500 U/ml) and GM-CSF (800 U/ml) (both from Schering-Plough, International, Kenilworth, New Jersey). Medium was refreshed at day 6. To generate mature DCs at day 6 recombinant 10 ng/ml TNF-α (Cell Genix, Freiburg, Germany), 10 μg/ml prostaglandin E2 (Pharmacia & Upjoin, Puurs, Belgium), 5 ng/ml IL-1β (ImmunoTools, Friesoythe, Germany) and 15 ng/ml IL-6 (Cell Genix) were added. Both immature and mature DCs were harvested on day 8.

Mixed lymphocyte reaction (MLR)

The ability of the DCs to induce T cell proliferation was studied in an allogeneic proliferation assay. Briefly, $1x10^5$ freshly isolated allogeneic PBMCs from a healthy donor were incubated with $5x10^3$ immature or mature DCs. After 4 days of culture, 1 μCi/well of tritiated thymidine was added per well. Tritiated thymidine incorporation was measured in a beta-counter after 8 hours of pulsing. MLR index is defined as thymidine incorporation with DC-stimulation divided by the thymidine incorporation when medium was added to the PBMCs. Cytokine production was measured after 24 hours by cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen).

Migration assay

We used our previously described *in-vitro* migration assay to study migration capacity of the DC subtypes.[12] Briefly, 3000 DCs (50 μl) per well were seeded on fibronectin coated flat bottomed plates, resulting in 100 cells per image. DCs were recorded for 1 hour, after which migration tracks of individual DCs were analyzed. Speed is defined as the traversed path during the entire experiment divided by the imaging time.

Statistical analysis

Data are presented as median ± range. The statistical significance of data was calculated using the Mann-Whitney U Test. All statistical tests were two-sided, significance was determined as $p<0.05$.

Results

Study group

DCs were cultured from fifteen pediatric patients who were newly diagnosed and had untreated tumors. As outlined in table I, the patients formed a heterogeneous group with several types of malignancies, both localized and metastasized. The median age of the patients was 10 years (range 1 to 16). The pediatric control group had a median age of 8 years (range 1 to 16) and a similar sex-distribution.

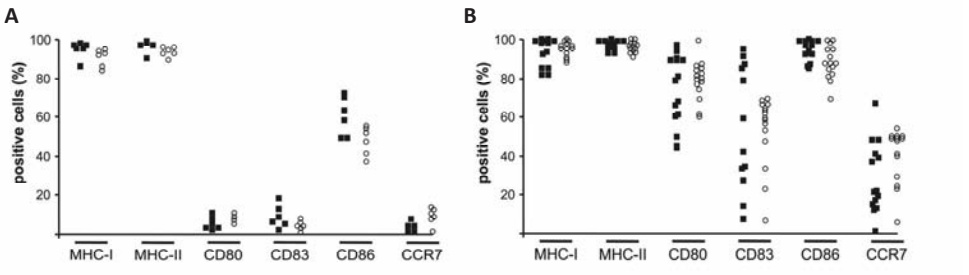
Table I. Clinical data of children with newly diagnosed cancer

Patient	Sex	Age (years)	Diagnosis	Disease status	WBC ($x10^6$/ml)
1.	M	14	Hodgkin lymphoma	Localized	15.2
2.	M	4	Rhabdomyosarcoma	Localized	8.2
3.	F	2	Rhabdomyosarcoma	Localized	6.6
4.	F	2	Neuroblastoma	Metastasized	5.5
5.	F	12	Neuroblastoma	Metastasized	9.4
6.	M	1	Ewing's sarcoma	Metastasized	9.8
7.	F	14	Ewing's sarcoma	Metastasized	10.0
8.	M	13	Ewing's sarcoma	Localized	7.4
9.	M	10	Osteosarcoma	Localized	8.0
10.	F	12	Osteosarcoma	Localized	10.3
11.	F	15	Osteosarcoma	Localized	8.4
12.	F	16	Osteosarcoma	Localized	8.5
13.	F	4	Wilms tumor	Metastasized	13.2
14.	F	3	Wilms tumor	Metastasized	10.6
15.	F	7	Dysgerminoma	Metastasized	5.7

PBMC isolation and DC culture

The median PBMC numbers derived from pediatric cancer patients and controls were respectively 2.4 and 2.7 $x10^6$ PBMCs/ml (table II). In approximately 50% of all patients sufficient DCs could be obtained to directly compare mature and immature DCs from the same donor. On average 1.4 $x10^5$ DCs/ml could be cultured from the mononuclear cell fraction, which is significantly lower compared to pediatric controls (table II). Within the group of pediatric cancer patients no correlation was observed between clinical stage and the yield of PBMC and DC numbers.

Figure 1



The phenotype of dendritic cells (DCs) analyzed by flow cytometry. The symbols represent the results obtained from individual pediatric cancer patients (closed squares) and pediatric controls (open circles). (a) Phenotype of immature DCs. (b) Phenotype of mature DCs.

DC phenotype of pediatric cancer patients

The immature DCs of pediatric cancer patients are characterized by a low expression of CD80, CD83 and CCR7 and a high expression of MHC-I and MHC-II (figure 1a). Upon DC-maturation a more heterogeneous population can be observed. Up-regulation of CD80, CD83, CD86 and CCR7 occurs in all cases. The phenotype of DCs from pediatric patients resembles to that from the controls (figure 1b). Changes in DC phenotype that occur during the process of DC maturation in the pediatric patients resemble those described for adult patients.[5]

Table II. Yield of mononuclear cells and dendritic cells

Study group	WBC ($x10^6$)[a]	PBMC ($x10^6$)[a]	DC ($x10^5$)[a]
Pediatric patients (n=15)	8.5 (5.5-15.2)	2.4 (1.6-5.4)	1.4 (0.7-2.6)[b]
Pediatric controls (n=15)	6.7 (3.8-15.1)	2.7 (1.4-6.3)	2.8 (1.1-4.0)

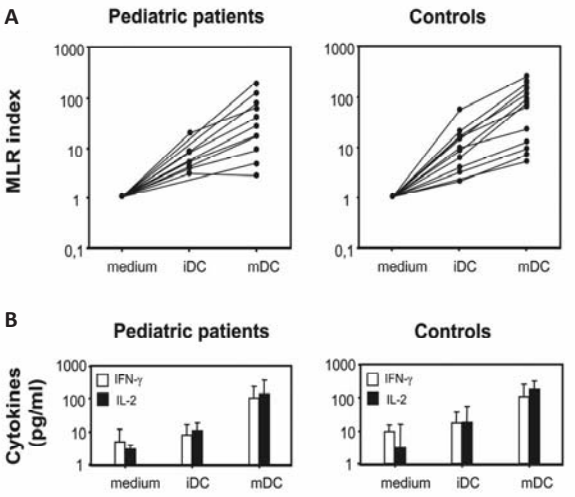
[a]Median yield per ml whole blood (range)

[b]DC yield is significantly lower compared to controls ($p<0.05$)

T cell proliferation induced by DCs

We tested the capacity of the DCs to induce T cell proliferation in a mixed lymphocyte reaction. Figure 2a demonstrates that mature DCs are on average six times more potent in inducing T cell proliferation than immature DCs. No statistically significant differences are observed between pediatric patients and controls. The potency of mature DC to stimulate T cell proliferation is further demonstrated by the cytokine production during the MLRs. T cells stimulated by immature DCs produced some IFN-γ and IL-2 whereas mature DCs induced high production of IFN-γ and IL-2 (figure 2b).

Figure 2

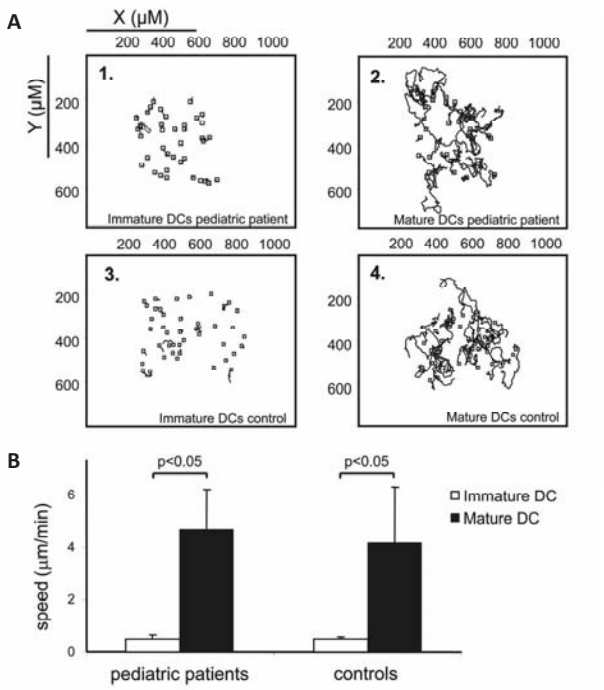


Ability of dendritic cells (DCs) to stimulate allogeneic lymphocytes in a mixed lymphocyte reaction. DCs from pediatric cancer patients are compared with controls. (a) Results are expressed as MLR index (see methods). The lines connect results obtained with medium alone, immature DCs and mature DCs from one patient. (b) Cytokine production. The white bars represent IFN-γ secretion (pg/ml) and the black bars represent IL-2 secretion (pg/ml). Both IFN-γ and IL-2 are measured after 24 hours (median values with range). No significant differences between pediatric patients and controls were observed ($p>0.05$).

In-vitro migration of DCs

We compared the adhesive and migratory capacity of immature versus mature DCs of 4 pediatric patients and 6 controls, using an *in-vitro* migration assay on fibronectin. In contrast to immature DCs, mature DCs were highly motile and showed random migration. Migration paths and speed of the DC subtypes are shown in figure 3a and 3b, respectively. Although cell membranes of immature DCs were highly dynamic, changing shape continuously, hardly any migration was observed (0.4 µm/min in pediatric patients and controls). In contrast, mature DCs from pediatric patients migrated at a median speed of 4.7 µm/min, which was statistically not different from pediatric controls (4.2 µm/min).

Figure 3



Migration of dendritic cells (DCs) on fibronectin. (a) Traversed paths of immature and mature DCs from a representative pediatric cancer patient (tracks 1 and 2) and a representative pediatric control (tracks 3 and 4). Each square represents one DC. (b) Median migration speed is calculated from pediatric patients (n=4) and controls (n=6). Statistically significant differences between immature and mature DCs migration speed are indicated by horizontal bars.

Discussion

Here we show that monocyte derived, fully mature DCs can be generated from blood of pediatric cancer patients. Secondly, the phenotype and function of these DCs parallels that of DCs generated from age-related controls. Thirdly, we demonstrate that DCs can be generated from the blood of pediatric patients in sufficient quantities to perform vaccination studies and that these DCs meet the release criteria for vaccination.[26]

Clinical studies in which tumor-antigen loaded DCs are used to vaccinate adult cancer patients indicate that mature DCs are superior to immature DCs in inducing anti-tumor responses.[14,15] This prompted us to explore the feasibility to generate high quantities of functional mature DCs from pediatric patients with cancer. We cultured the DCs, as most clinical studies to date, from monocytes in IL-4 and GM-CSF.[26] DCs were matured with a clinical grade cocktail of TNF-α, prostaglandin E2, IL-1β and IL-6.[27] Vakkila et al. already demonstrated that viable immature DCs can be cultured from monocytes of pediatric patients. Both phenotype and function of these immature DCs were equal to immature DCs cultured from age related controls, however yield was lower in pediatric cancer patients.[28] Pospisilova et al. demonstrate that the immature DCs from acute lymphoblastic leukemia pediatric patients obtain a slightly matured phenotype when they are pulsed with tumor lysate.[29]

We found that the presence of an untreated pediatric malignancy was correlated with a reduced number of DCs that could be generated from the peripheral blood ($1.4x10^5$ mature DCs/ml blood). These data are comparable to the culture efficiency of immature DC in children with cancer.[19,28] The lower yield of DCs in pediatric patients compared to controls is correlated with the malignancy and not the age, since we found no difference in DC yield between pediatric non-malignant controls and adult healthy controls (data not shown).

In an allogeneic MLR we showed that monocyte derived mature DCs from pediatric patients are six times more potent in inducing T cell proliferation than immature DCs. The efficiency of mature DCs to induce T cell stimulation is further demonstrated by the production of high amounts of IFN-γ and IL-2 (figure 2b). In addition, mature DCs of pediatric patients, but not immature DCs, have the ability to migrate *in-vitro*. Furthermore, mature DCs also express CCR7 which is absent on immature DCs. The ligands for the CCR7 receptor, the chemokines 6Ckine and MIP-3β, are primarily produced in the T-cell rich parafollicular areas of lymph nodes.[30-32] Hence monocyte-derived immature DCs of pediatric patients generated *in-vitro* may less efficiently migrate to and interact with naive T cells in the lymph nodes; a prerequisite for the induction of an effective immune response.

In summary, we conclude that the presence of untreated malignant disease of pediatric patients significantly reduced the number of DCs that could be cultured from monocyte precursors, but did not affect their phenotype and function. From pediatric patients, the *ex-vivo* generated monocyte derived mature DCs, and not immature DCs, have the capacity to migrate and induce a strong proliferative response *in-vitro*. All together these data indicate that sufficient quantities of clinical grade mature DCs can be generated and used in autologous DC vaccination trials in pediatric cancer patients.

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Chapter 3

Targets for active immunotherapy against pediatric solid tumors

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Abstract

The potential role of antibodies and T lymphocytes in the eradication of cancer has been demonstrated in numerous animal models and clinical trials. In the last decennia new strategies have been developed for the use of tumor-specific T cells and antibodies in cancer therapy. Effective anti-tumor immunotherapy requires the identification of suitable target antigens. The expression of tumor-specific antigens has been extensively studied for most types of adult tumors. Pediatric patients should be excellent candidates for immunotherapy since their immune system is more potent and flexible as compared to that of adults. So far, these patients do not benefit enough from the progresses in cancer immunotherapy, and one of the reasons is the paucity of tumor-specific antigens identified on pediatric tumors. In this review we discuss the current status of cancer immunotherapy in children, focusing on the identification of tumor-specific antigens on pediatric solid tumors.

Introduction

Despite major advances in the treatment of childhood malignancies, cancer remains in the developed world the second most common cause of death for children > 1 year of age.[1] Children and adolescents with primary multifocal, refractory or relapsed malignant solid tumors still have a poor prognosis. Moreover, most cancer therapies are associated with significant toxicity leading to long-term morbidity and an increased second malignancy rate.[2,3] Therefore, new treatment strategies are warranted. One of them is immunotherapy, in which the patient's own immune system is mobilized to fight the cancer in a specific way, thereby causing only mild toxicity.[4]

The immune system can reject tumors

Early studies in mice showed that the immune system can recognize and reject tumors.[5] Numerous mouse tumor models have been developed to identify which part of the immune system is responsible for the eradication of tumors. These studies indicate that both $CD8^+$ and $CD4^+$ T cells play a critical role in tumor rejection or in inhibition of tumor growth.[6] The cytolytic activity of $CD8^+$ T cells exerts a direct anti-tumor effect.[7] $CD4^+$ T cells participate through the activation and maintenance of $CD8^+$ T cells and the recruitment of inflammatory cells such as macrophages, granulocytes, NK cells and B cells.[8-12]

In humans, it has occasionally been observed that primary tumors as well as metastases, for example metastasized neuroblastomas in children, can spontaneously disappear.[13] Although there are no known immune mechanism for metastasized neuroblastomas to spontaneously regress, this suggests that immune-mediated mechanisms may control this disease in children.[14,15]

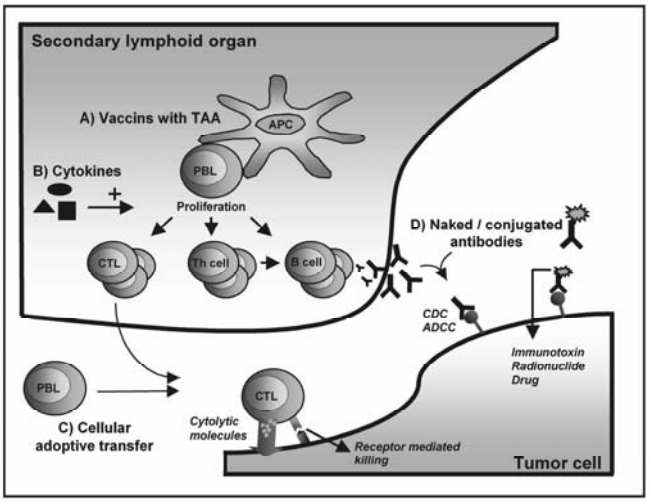
Tumor-infiltrating immune cells have frequently been observed in a wide variety of pediatric tumors.[16,17] Tumor infiltration of lymphocytes is generally associated with a more favorable prognosis and occasionally tumor regression.[18,19] Another element is the observation that immunosuppressed patients, such as graft recipients, are at higher risk to develop cancer.[20,21] Initial studies have consistently shown a role of the immune system in the prevention of virally induced cancers in adults such as Kaposi's sarcoma (linked with human herpes virus 8), cervix carcinoma (human papilloma virus) and hepatocellular carcinoma (hepatitis B and C)[22] but also in children with certain lymphomas (induced by the Epstein-Barr virus).[23,24] More recent studies of transplant recipients showed also a significant increase of cancers without viral aetiology such as carcinomas of the lung, colon, bladder, prostate, kidney and skin.[25-27] These data suggest that the immune system plays an important role in preventing or controlling malignancy in both adults and children. In spite of the blood-brain-barrier and lack of conventional lymphatics in the brain, there is accumulating evidence that even brain tumors can cause immune activation.[28-30]

Immunotherapy strategies in pediatric cancer patients

Immunotherapy can be defined as any approach that seeks to mobilize or manipulate the immune system of a patient for therapeutic benefit (figure 1).[31,32] Clinical experience of immunotherapy in the pediatric oncological setting has been gained in

treating hematologic malignancies with allogeneic bone marrow transplantations and infusions of donor lymphocytes to generate graft versus leukemia responses.[33] Other clinical trials for pediatric patients have involved general immunostimulation with cytokines such as IL-2, TNF-α and IFN-α, as adjuvant therapies to eradicate minimal residual disease.[34-37] Immunotherapeutic therapies targeting identified tumor associated antigens are discussed below.

Figure 1



Immunotherapeutic strategies applied in pediatric clinical trials. A) administration of tumor antigens either directly into the body or loaded onto APC. The TAAs are presented by the APC to lymphocytes in secondary lymphoid organs to initiate a tumor-specific immune response. B) Non-specific stimulation of the immune response by cytokines, for example IL-2, TNF-α and IFN-α and GM-CSF which induces T cell proliferation. C) Adoptive transfer of donor lymphocytes or natural killer cells for complete eradication of leukemic cells following allogeneic transplantation. D) Monoclonal antibodies (mAb) that bind specifically to cancer cells can induce an immune response. Alternatively, mAb can be modified for targeted delivery of a toxin, radioisotope, cytokine or other active conjugate. TAA, tumor associated antigens; APC, antigen presenting cell; PBL, peripheral blood lymphocyte; CDC, complement dependent cytotoxicity; ADCC, antibody dependent cell-mediated cytotoxicity.

Antibodies

The identification of tumor-specific cell-surface molecules opened the possibility for antibody-mediated passive immunotherapy. Antibodies (Ab) against tumor associated antigens can induce complement dependent cytotoxicity (CDC) and Ab-dependent cell-mediated cytotoxicity (ADCC).[38] Promising pediatric clinical phase I trials have been described using monoclonal Ab against gangliosides, which are highly expressed in neuroblastoma and osteosarcoma.[39-42] Tumor-specific Ab conjugated to toxins are under investigation as targeted drug-vehicles for embryonal tumors.[43,44]

Adoptive cellular immunotherapy

Reconstituting or increasing cellular immunity can be achieved through the infusion of tumor-specific T cells. Autologous $CD4^+$ or $CD8^+$ T cells can be manipulated ex vivo in various ways to obtain high numbers of clinical grade tumor-specific T cells.[45] The therapeutic effect of infused tumor-specific T cells depends on the viability of the cells, their homing to the tumor, and their ability to kill within the tumor microenvironment.

Another aspect is the renewed appreciation of the role of the innate immune system. Immune-mediated tumor lysis is the result of a combined action of adaptive and innate immunity, in which natural killer (NK) cells are important effector cells. NK cell activation is regulated by a balance between signals mediated through activating receptors such as NKG2D and inhibitory receptors such as killer immunoglobulin-like receptors (KIRs). Upon cellular transformation in tumor cells, MHC class I ligands for inhibitory receptors are often downregulated and ligands for activating NK cell receptors are upregulated on the tumor cell. Together, these events can shift the balance towards NK-mediated tumor-cell killing.[46] Next to the direct cytotoxic effect on tumor cells, natural killer cells produce type I interferons that create a proinflammatory tumor microenvironment.[47,48] Clinical studies on adoptive transfer of natural killer cells in adults have shown that natural killer cells can have a role in the treatment of selected malignancies.[49] Adoptive transfer of NK cells in pediatric patients with leukemia is feasible.[50] Ongoing clinical studies further investigate NK cell mediated immunotherapy for pediatric patients with leukemia or neuroblastoma (*http://www.clinicaltrials.gov*).

In vivo induction of tumor-specific lymphocytes

The advantage of active immunization over adoptive transfer is the possibility of inducing memory T-cells that can control tumor relapse.[51] On the basis of the successes of attenuated pathogen vaccines and owing to the initial lack of defined tumor antigens, the first active immunizations were carried out with whole tumor cells that were previously irradiated or otherwise inactivated.[52] In children, most of the clinical experience using whole tumor cell vaccines is obtained with neuroblastoma patients. In these trials, the neuroblastoma cells are (gene-)modified to express various co-stimulatory molecules or cytokines to increase their immunogenicity.[53-55]

When tumor associated antigens are identified, therapeutic vaccination can involve the administration of the antigen either as a whole recombinant protein or as antigenic peptides presented by HLA class I or class II molecules. One clinical trial reports on using chimeric antigenic peptides encoded by translocated genes expressed in Ewing's sarcoma and rhabdomyosarcoma.[56] Another strategy is the administration of autologous antigen presenting cells, such as dendritic cells, loaded with defined tumor antigens or with tumor cell lysates. We reported that clinical grade dendritic cells can be cultured from blood monocytes of pediatric cancer patients.[57] Others have reported that such dendritic cells can induce tumor-specific T-cells that can cause regression of high risk malignancies in pediatric patients.[58-61]

Advances in gene transfer technology have added new possibilities to optimize vaccine preparation.[62,63] These include transferring genes encoding pro-inflammatory proteins to tumor cells and transferring tumor antigen encoding genes into

professional antigen-presenting cells. Tumor cells can be engineered to express MHC class I and class II, costimulatory molecules, or cytokines, and used as vaccines. Several gene therapy applications to induce antitumor immunity have been reported for pediatric cancer patients in preliminary phase I studies.[53]

Current research also focuses on vaccinating directly with antigen-encoding DNA. Studies in animal models have demonstrated the feasibility of utilizing DNA vaccines to elicit protective cellular and humoral antitumor immune responses.[64] In humans, DNA vaccines are being tested in phase I to III clinical trials for cervical cancer, melanoma, renal cell carcinoma and prostate cancer.[65] Preliminary results confirm the safety and immunogenicity of these vaccines. DNA vaccinations have not been studied in pediatric patients. However, first steps are taken with murine studies showing that DNA-vaccination is potentially effective to treat neuroblastoma and prevent neuroblastoma metastases.[66,67]

Tumor associated antigens

One of the reasons for the paucity of clinical trials of therapeutic anti-cancer vaccination in children is the lack of information about the expression of tumor-specific antigens on many pediatric tumors. In the second part of this review we will summarize the current data on the expression of tumor antigens recognized by T cells on a selection of the most common solid pediatric tumors.

Tumor antigens that can be recognized by T lymphocytes are complexes of HLA class I or class II molecules presenting small antigenic peptides. The antigens can be classified into four major groups, based on the pattern of expression of the genes encoding the antigenic peptide.[68,69]

Antigens resulting from mutations or translocations

These antigens are encoded by genes that are mutated in tumor cells as compared to the normal cells of the patient, the antigens can therefore be considered strictly tumor specific. The mutations can be point mutations, or translocations, in genes that are expressed ubiquitously. The mutation affects a coding region of the gene, and antigenic peptides contain mutated residues or straddle the junction of chimeric proteins encoded by translocated genes.

Antigens encoded by cancer-germline genes

Cancer-germline genes are expressed in different types of human tumors. They are not expressed in normal tissues with the exception of male germline cells which do not express HLA molecules and therefore cannot present antigenic peptides to T cells.[70] For this reason the antigens encoded by cancer-germline genes are strictly tumor-specific.

Differentiation antigens

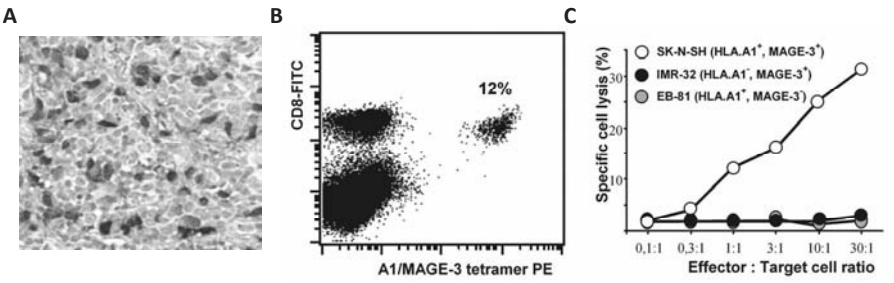
Differentiation antigens are encoded by lineage-specific genes that are expressed in tumor cells as well as in the normal cells from which the tumor arises. The natural

tolerance to these antigens is not complete, and the induction of an immune response against differentiation antigens is possible.[71]

Antigens encoded by genes that are overexpressed in tumors
This last group of tumor antigens is encoded by genes that are overexpressed in tumors as compared to normal tissues. Some oncogenes are expressed in normal tissues at a low level and overexpressed in several tumors.[72,73] Since both differentiation antigens and overexpressed antigens are expressed in normal tissues, autoimmunity can be a side-effect when these antigens are used as immunotherapeutic target.

T cell defined antigens in pediatric solid tumors
For usefulness in immunotherapy, an antigen has to meet two important criteria. It has to be expressed by the tumor of the patient, and it has to be immunogenic. These criteria can be tested with gene/protein expression and lymphocyte recognition/tumor lysis assays (figure 2). For an effective cellular immune response, the tumor-specific antigen must be processed into peptides and presented on HLA molecules. Many tumor associated antigens and epitopes have been described that are recognized by $CD4^+$ and/or $CD8^+$ T cells. Detailed lists of antigen-encoding genes and of epitopes can be found at http://www.cancerimmunity.org.

Figure 2



In vitro assays to assess target suitability. A) Immunohistochemistry of MAGE-1 expression in a neuroblastoma tumor (antibody MA454) demonstrates the heterogeneous expression of MAGE-1 in this tumor sample. B) Visualization of MAGE-3 specific $CD8^+$ cells using labeled CD8-Ab and A1/MAGE-3-tetramers. Dot plot of peripheral blood mononuclear cells from a patient who received a vaccine containing MAGE-3.A1 peptides. 12% of the $CD8^+$ cells are tetramer-positive after two weeks of in vitro re-stimulation with the MAGE-3.A1 peptide (EVDPIGHLY).[97] C) Chromium release assay using cytotoxic T-lymphocyte clone EH-1 B2.C10, which recognizes peptide MAGE-$3^{168-176}$ presented by HLA-A1 molecules. Lysis was tested after 4 h at 37°C, as previously described.[98] Only the HLA-$A1^+$, MAGE-3^+ SK-N-SH neuroblastoma cell line (white dots) is efficiently lysed by the CTL clone. Cell lines that are either HLA-A1 negative (IMR-32, black dots) or do not express gene MAGE-3 (EB81-EBV-B, grey dots) are not lysed.

Tables 1 and 2 summarize T-cell defined antigens on a selection of the most important pediatric solid tumors. The antigens are categorized according to the four groups mentioned in the previous paragraph. To produce a clinically relevant list, we have included only antigens of which 1) peptides recognized by T cells are identified, 2) the HLA presenting molecule is identified, 3) evidence exists that the peptide is processed and presented by tumor cells and 4) a certain level of tumor- or tissue-specificity is reported. Virus-encoded and artificially modified epitopes are excluded from this list. Antigens of solid tumors outside the central nervous system are shown in Table 1, and those of brain tumors in Table 2. The percentages indicate the proportions of tumors expressing the gene, tested with RT-PCR or immunohistochemistry. Original papers are only referred to if expression has been investigated in at least 10 histologically similar tumors, with no restriction as to the year of publication.

All tumors reviewed here, except neuroblastoma, also occur in adults. Most papers about antigen expression do not report whether tumor samples are derived from adults or children. Only a few papers specifically report on antigen expression in pediatric tumors.[74-80] It is important to note that the expression of a given antigen in tumors of adult patients does not guarantee that this antigen is also expressed in the tumor of that same subtype from a pediatric patient. We and others observed significant age-related differences in the expression of tumor antigens in glioblastoma samples.[76,80-82] For some antigens we noticed important differences in the expressions reported by different groups. They can be due to the sensitivity/specificity of the techniques used (microarray, RT-PCR and immunohistochemistry), to different antibodies or primer-pairs for the same antigen, and to differently chosen cut-off points.

Table 1. T cell defined antigens in extra-cranial pediatric solid tumors[a]

Antigen [refs]	neuroblastoma	rhabdomyosarc.	osteosarc.	Ewing's sarc.
Antigens from fusion proteins:				
PAX3/FKHR [99]	0%	60%[b]	0%	0%
EWS/FLI 1 [100]	0%	0%	0%	85%
Cancer-germline genes:				
GAGE [101,102]	82%	9-16%	N.D.	100%
MAGE-1 [75,102-106]	18-60%	25-38%	55-88%	0%
MAGE-2 [75,102,106,107]	60-61%	33-51%	55-78%	0%
MAGE-3 [102-106]	33-76%	35-42%	52-100%	28%
NY-ESO-1 [75,105,108]	36-82%	25%	88%	0%
Overexpressed antigens:				
HER-2 [109-114]	14%	11%	0-44%	0%
MYCN [115-117]	20-25%	43-60%[b]	N.D.	N.D.
P53 [109,113,118-122]	84%	19-67%	14-27%	11-43%
Survivin [123-125]	47-54%	N.D.	58%	N.D.

[a]The percentages indicate the proportion of tumors expressing the gene, tested with RT-PCR or IHC.
[b]expression in alveolar rhabdomyosarcoma; no expression of MYCN in embryonal rhabdomyosarcoma.
GAGE, G antigen; HER-2, human epidermal receptor 2; MAGE, melanoma-associated antigen; N.D., not determined; NY-ESO-1, New York esophagous 1; P53, protein 53; WT-1, Wilms' tumor 1 gene.

Table 2. T cell defined antigens in pediatric brain tumors[a]

Antigen [refs]	low grade astrocytoma	high grade astrocytoma	ependymoma	medulloblastoma
Cancer-germline genes:				
GAGE [126]	N.D.	11%	43%	13%
MAGE-1 [76,126-129]	0-33%	0-100%	0%	9-13%
MAGE-2 [76,126]	12-18%	10-11%	57%	18-60%
MAGE-3 [76,126,128,130]	18-35%	20-33%	0-33%	13-18%
NY-ESO-1 [76,130]	0-14%	0-10%	N.D.	9%
Overexpressed antigens:				
HER-2 [74,131-133]	0-77%	5-93%	83%	38-86%
IL-13R [77,134]	79%	100%	67%	67-100%
MYCN [135-138]	N.D.	43%	N.D.	5-21%
P53 [78,79,139-141]	8-72%	52-63%%	28-48%	17-27%
Survivin [80,142-144]	37-64%	80-92%	100%	100%
WT-1 [145]	40%	56%	56%	39%
Differentiation antigens:				
Tyrosinase [128]	67%	38%	50%	N.D.
Gp100 [128,132]	33%	38-47%	50%	N.D.

[a]The percentages indicate the proportion of tumors expressing the gene, tested with RT-PCR or IHC. GAGE, G antigen; gp100, glycoprotein 100; HER-2, human epidermal receptor 2; IL, interleukin; MAGE, melanoma-associated antigen; N.D., not determined; NY-ESO-1, New York esophagous 1; P53, protein 53; WT-1, Wilms' tumor 1 gene.

Which antigens to choose for pediatric clinical trials?

Table 1 and 2 list T cell defined antigens expressed on pediatric tumors that can be used as immune target in clinical trials. So far, the antigens have primarily been used in clinical trials in adult patients with the exception of clinical trials in pediatric patients targeting the following antigens: PAX3/FKHR and EWS/FLI1,[56,83] WT-1 (ongoing clinical trial, http://www.clinicaltrials.gov) and MAGE-A1 (Jacobs et al, manuscript in preparation). Choosing the best antigen in a specific immunotherapy trial depends on the individual needs for that study such as the immunogenicity of the antigen, the level of antigen expression by the tumors, the tumor-specificity of the antigen, the availability of clinical grade antigenic products, and the HLA-type of the included patients.

Mutated tumor antigens are attractive antigens for cancer immunotherapy because of their strict tumor specificity and because of their potential resistance to immunoselection when the mutated gene product plays an important role in the oncogenic process. A drawback is that most point mutations, in contrast to chromosomal translocations, are not shared by tumors from different patients. Examples of chimeric proteins in the pediatric setting are the PAX3-FKHR, EWS-FLI 1, TEL-AML1 and BCR-ABL fusion proteins seen in alveolar rhabdomyosarcoma, Ewing's sarcoma, acute lymphatic leukemia and chronic myeloid leukemia, respectively.[84-87] For all four fusion-proteins several MHC class I and class II chimeric peptides have been described that induce specific T cells and can be considered for immunotherapy.[83,84,88,89]

The other genetic mechanism responsible for tumor specificity of antigens is the aberrant expression in tumor cells of genes that are silent in normal cells. When the antigens are encoded by genes that are expressed in many different tumors they are called 'shared tumor-specific antigens'. Most of the shared tumor-specific antigens are encoded by *cancer-germline genes.* Cancer-germline genes such as MAGE, GAGE or LAGE/NY-ESO-1, are expressed in different types of pediatric tumors (Tables 1 and 2). Numerous peptides, binding to different HLA class I and HLA class II molecules have been identified.[90] Because of their tumor-specificity and immunogenic potential, antigens encoded by cancer-germline genes have been one of the main components of antitumor vaccines tested in the clinic during the last decade.[70]

Approximately 20% of all identified tumor antigens are encoded by genes that are overexpressed in cancer cells as compared to normal cells. Overexpression in this context means more antigenic peptides presented on MHC molecules at the cell surface, explaining the tumor-specificity of the T lymphocytes. As shown in Tables 1 and 2, many of the identified antigens in pediatric solid tumors are classified as *overexpressed antigens*. HER-2, WT-1 and MYCN are the most interesting candidates for immunotherapy since these genes are involved in cell proliferation and their overexpression plays a direct functional role in tumor progression. This role in oncogenesis implies that it is more difficult for the tumor to escape immune attack through downregulation of antigen expression. The absence of autoimmune tissue damage in cancer patients with HER-2, WT-1 or MYCN specific CTLs suggests that these antigens can be safely used as immunotherapeutic target.[91-93]

With the observation that tumor-specific CTL clones derived from melanoma patients could also recognize normal melanocytes it became obvious that natural tolerance to differentiation antigens was incomplete.[94] Gp100 and tyrosinase are the only *differentiation antigens* expressed in pediatric tumors for which T cell specific peptides are identified (see Table 2). Autoimmunity can be a side-effect when differentiation antigens are used for vaccination. Since gp100 and tyrosinase are expressed in normal melanocytes, it is possible that pediatric patients will develop vitiligo when these antigens are used in a vaccine.[95,96]

For safety concern, the target antigens used in pediatric clinical trials should be strictly tumor-specific. If such an antigen is not available, the normal tissue expressing the antigen must be dispensable, to avoid serious autoimmune toxicity. Finally, it is probably preferable to use combinations of antigens to decrease the probability of in vivo selection of antigen-negative tumor cells.

Conclusion

Immunotherapy against cancer is a field of growing interest. Most therapies are still experimental and focus on adult patients. However, the first immunotherapeutic trials for pediatric cancer patients have been published, and more are ongoing. These novel trials aim at stimulating both humoral and cellular anti-tumor immune responses. The identification of many tumor associated antigens, including for most pediatric solid tumor types, should facilitate this clinical endeavour.

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Chapter 4

Cancer-germline gene expression in pediatric solid tumors using quantitative real-time PCR

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ABSTRACT

Cancer-germline genes (CGGs) code for immunogenic antigens that are present on various human tumors but not on normal tissues. The importance of CGGs in cancer immunotherapy has led to detailed studies of their expression in a range of human tumors. We measured the levels of expression of 12 CGGs in various pediatric solid tumors to identify targets for therapeutic cancer vaccines.

Quantitative real-time PCR (qPCR) was used to measure the expression of 8 *MAGE* genes and of genes *LAGE-2/NY-ESO-1* and *GAGE-1,2,8* in 9 osteosarcomas, 10 neuroblastomas, 12 rhabdomyosarcomas and 18 Ewing's sarcomas. Nine tumors were also examined by immunohistochemistry with monoclonal antibodies specific for the MAGE-A1, MAGE-A4 and NY-ESO-1 proteins.

All osteosarcoma and 80% of neuroblastoma samples expressed several CGGs at high levels. Six of 12 rhabdomyosarcomas and 11 of 18 Ewing's sarcomas expressed at least one CGG. Immunohistochemistry data correlated well with qPCR results and showed a homogeneous protein distribution pattern in most positive tumors. No correlation was found between the levels of CGG expression in the tumors and clinicopathological parameters of the patients.

Pediatric solid tumors express several CGGs which encode antigens that could be targeted in therapeutic vaccination trials. Several CGGs of the *MAGE, GAGE* and *LAGE* families, are co-expressed in a large proportion of osteosarcoma and neuroblastoma samples. Some rhabdomyosarcomas express several of these genes at high levels. Ewing's sarcomas have an overall low CGG expression.

INTRODUCTION

Human tumors bear antigens that can be specifically recognized by autologous T lymphocytes and antibodies.[1-3] It has been shown in animal models and in clinical trials that vaccination with these tumor-specific antigens can lead to the eradication of tumors.[4,5] Over the last decade, promising new treatment modalities of induction of tumor-specific T cells have been proposed and applied.[6,7]

Cancer-germline genes (CGGs) are expressed in male germline cells, not in other normal adult tissues, and in many tumors. Because male germline cells do not express HLA molecules on their surface, they do not express antigens that can be recognized by T cells. As a result, the antigens encoded by CGGs are strictly tumor-specific T cell or antibody targets. These antigens are shared by many tumors. CGGs include the MAGE,[8-10] GAGE,[11,12] and LAGE/NY-ESO-1[13,14] families. The MAGE gene family comprises 24 genes ranged in three subfamilies, MAGE-A, -B, and -C. A large number of antigenic peptides encoded by the MAGE, GAGE and LAGE/NY-ESO-1 genes have been found to be presented to CD4 and CD8 lymphocytes by HLA molecules expressed at the surface of tumor cells.[15] Several clinical trials in which antigens encoded by CGGs were used as vaccines have shown induction of immunological responses and, in a small number of patients, clinical responses.[16-22]

Immunotherapy is an attractive therapeutic modality for pediatric cancer patients because it has a very mild toxicity and the immune system of these patients is more potent and flexible compared to adults.[23,24] However, the development of appropriate vaccines has been hampered by the lack of knowledge regarding expression of tumor-specific antigens on pediatric tumors.

The aim of this study was to analyse the expression of CGGs in pediatric extracranial solid tumors. We report the results of a quantitative real-time PCR analysis of the expression of 12 CGGs in a panel of pediatric neuroblastomas, Ewing's sarcomas, rhabdomyosarcomas and osteosarcomas. For a subset of tumors, immunohistochemistry was used to analyse also the expression and distribution of proteins encoded by three CGGs.

MATERIAL AND METHODS

Tumor samples

Fresh-frozen tumor samples were available at the Department of Pathology at the Radboud University Nijmegen Medical Centre. All samples were from pediatric patients (0 to 20-year-old) with a malignant solid extracranial tumor diagnosed at the Department of Pediatric Hemato-Oncology between 2000 and 2004. Sections of the frozen samples were stained with hematoxylin-eosin and reviewed by the pathologist to verify tumor histology and to evaluate the percentage of tumor cells. Samples were only considered for study if the contents of tumor cells was ≥ 80% and the yield of DNase-treated RNA was >1 µg.

RNA isolation and cDNA synthesis

Total RNA was isolated with TriZol reagent (Invitrogen, Carlsbad, CA) and samples were treated with Deoxyribonuclease I (Invitrogen) according to the manufacturer's protocol. To generate cDNA, 1 µg DNase-treated RNA was reverse-transcribed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo(dT) primer and 50 units SuperScript II, according to the manufacturer's protocol. After first-strand synthesis, samples were diluted to a final volume of 100 µl with water.

Conventional PCR

Duplex PCR amplification of β-actin and GAPDH transcripts was carried out in a 25-µl reaction volume containing 2.5 µl of cDNA, 1X PCR Buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 100 µM each dNTP, 0.4 µM each primer, and 0.625 units Taq DNA polymerase (TaKaRa, Shiga, Japan). β-actin primers were as described.[25] GAPDH primers (originally available from Clontech, Palo Alto, CA; kindly provided by Dr B. Lethé) were 5'-TgAAggTCggAgTCAACggATTTggT-3' (sense) and 5'-CATgTgggCCATgAggTCCACCAC-3' (antisense). Cycling was performed in a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) as follows: 94°C for 4 min, followed by 22 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. Cycling was concluded with a final extension step at 72°C for 15 min. PCR products were fractionated in 1.3% agarose gel and visualized by ethidium bromide fluorescence (β-actin, 626 bp; GAPDH, 983 bp). PCR amplification of MAGE-A transcripts was carried out with the primer pair designed by Zammatteo et al.[26] These primers derive from a consensus nucleotide sequence for the last exon of the 12 MAGE-A genes and give amplicons of ~539 bp. PCR conditions were as described above, except that PCR was performed for 30 cycles.

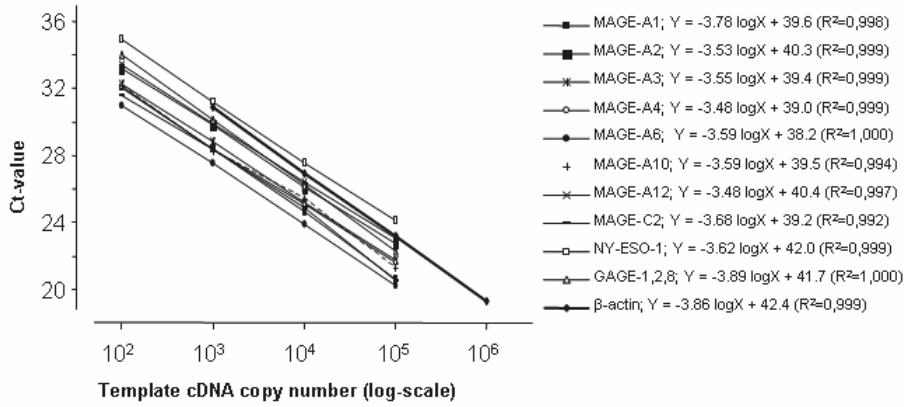
Quantitative real-time PCR

Expression of CGGs and of the reference gene β-actin, was measured by quantitative PCR, based on TaqMan methodology, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). PCR reactions were prepared with the qPCR Core Kit w/o dUTP reagents (Eurogentec, Seraing, Belgium). Each reaction (25 µl) contained 2.5 µl of cDNA, 1X PCR buffer containing the passive reference dye ROX, 5 mM $MgCl_2$, 200 µM each dNTP, 200 nM each primer, 100 nM probe, and 0.625 units DNA polymerase. Sequences of primers and probes, and thermal cycling conditions are

available from the authors. Probes with 6FAM and TAMRA labels were from Eurogentec. Probes with 6FAM and MGB-NFQ labels (for *MAGE-A3* and *MAGE-A6*) were from Applied Biosystems. Quantification of the samples was achieved by extrapolation from a standard curve of 4 serial dilution points of cDNA of the relevant gene (from 10^5 to 10^2 copies per reaction for CGGs, and from 10^6 to 10^2 copies per reaction for *ß-actin*). Samples and standard dilution points were assayed in duplicate or triplicate. Standard calibration curves for β-*actin* and all CGGs are shown in fig. 1. The assays were linear over 4 (CGGs) or 5 (*ß-actin*) orders of magnitude and had similar PCR efficiencies (slope from -3.48 to -3.89). Differences in sensitivity between the assays for the various genes (y-intercept from 38.2 to 42.4) were due in part to differences in the actual cDNA copy number in the standard dilutions. cDNA copy numbers in the standards were verified by testing minimally 12 replicates of the 1-copy dilution in each qPCR run. If needed, copy numbers of the test samples were corrected by a factor calculated on the basis of the results for the 1-copy dilution. Normalization of samples was achieved by dividing the copy number of CGG by that of the reference gene, β-*actin*.

Figure 1

A



Real-time qPCR: standard calibration curves for the 10 cancer-germline genes (CGGs) and the reference gene ß-actin. Ct values (y-axis) were plotted against the logarithm of template cDNA copy numbers (x-axis). The assays were linear over 4 (CGGs) or 5 (ß-actin) orders of magnitude.

Immunohistochemistry

Immunochemistry was performed on 4 µm tissue sections of formalin-fixed paraffin-embedded tissue blocks. Sections were boiled for 20 min in citrate buffer (10 mM, pH 6.0) for antigen retrieval. The following mouse IgG1 monoclonal antibodies (mAb) were used: E978 (anti-*NY-ESO-1*)[27] (Zymed, San Francisco, CA), MA454 (anti-*MAGE-A1*)[28] (Zymed), and 57B (anti-*MAGE-A4*)[29,30] (kindly provided by Dr. G.C. Spagnoli,

University Hospital Basel, Switzerland). Testis tissue with intact spermatogenesis was used as positive control. Tissue sections were incubated with mAb diluted in PBA (2.5 µg/ml E978, 1 µg/ml MA454, 5 µg/ml 57B), or with IgG1 negative control antibody, at room temperature for 1 hour. Primary antibodies were then detected with a biotinylated horse–antimouse secondary reagent (Vector Laboratories, Burlingame, CA) followed by an avidin–biotin complex system (ABC Elite, Vector Laboratories). Diaminobenzidine tetrachloride served as a chromogen. Immunoreactivity was assessed by the pathologist, blindly with respect to the mAb used.

RESULTS

Study population

We analysed the expression of cancer-germline genes (CGGs) in 49 freshly frozen tumors by reverse transcription and polymerase chain reaction (PCR) amplification. All samples were histologically proven malignant solid tumors of extra cranial origin, resected from patients ≤ 20 years old at the time of surgery (Table 1). The integrity of cDNA samples was verified by conventional, 22-cycles PCR amplification of 626 bp β-actin and 983 bp GAPDH products (data not shown). cDNA obtained from all 49 samples was then used as template in a conventional PCR amplification with consensus primers for the 12 genes of the MAGE-A family. Thirty-one samples scored positive, with 42-100% of positive samples depending on the type of tumor (Table 1).

Table I. Study group

Tumor type	Number of patients	Average age in years (range)	*% of MAGE-A*-positive tumors[c]
Neuroblastoma	10	1 (0.2-3)	80
Ewing's sarcoma	18	13 (2-20)	50
Rhabdomyosarcoma	12[a]	6 (0-20)	42
Osteosarcoma	9[b]	12 (9-16)	100

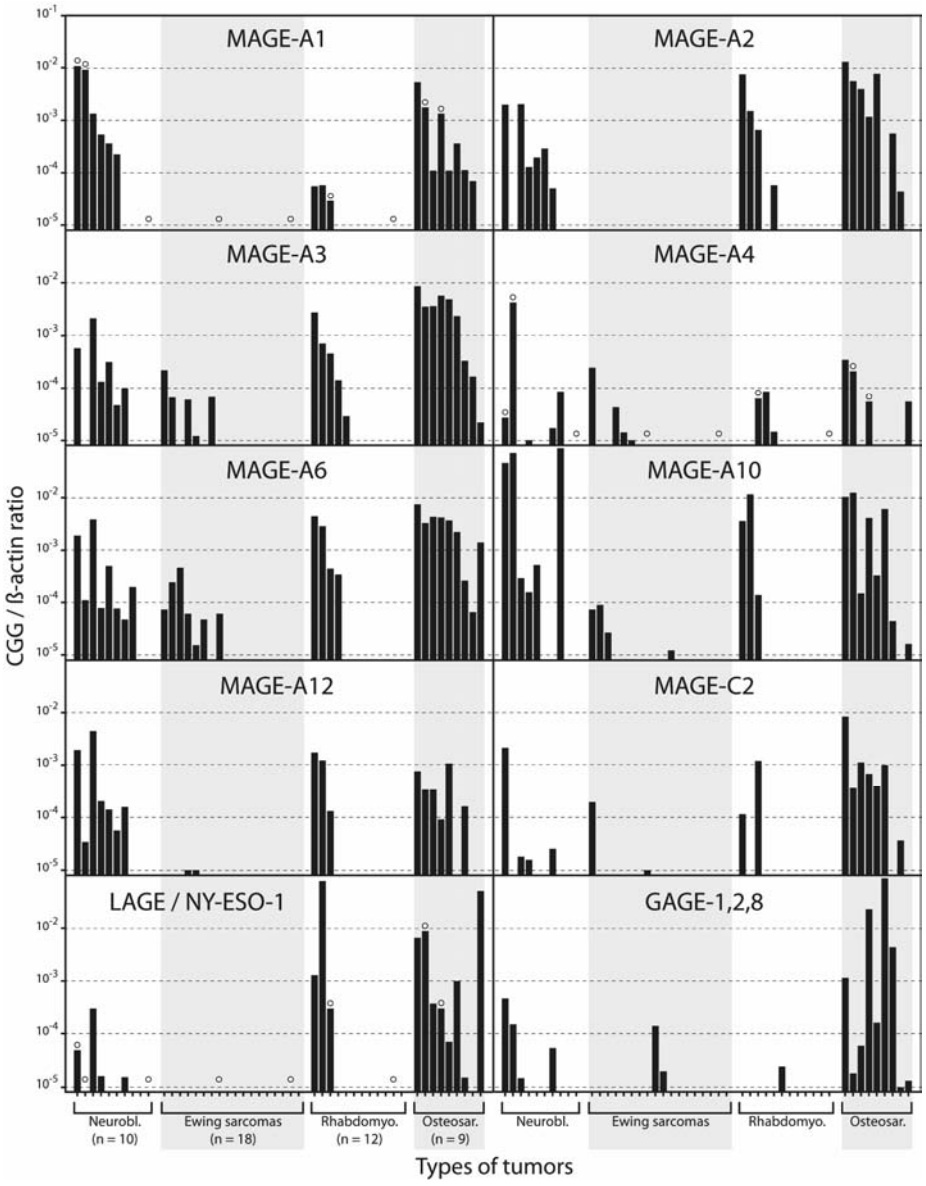
[a] 5 samples of alveolar and 7 of embryonic origin.
[b] 3 samples were obtained after chemotherapy, represented by osteosarcoma bars 5,6 and 8 in fig. 2.
[c] Gene expression determined by conventional PCR with consensus primers for the 12 genes of the *MAGE-A* family.

CGG expression in pediatric tumors

The 31 MAGE-A-positive samples were tested by quantitative real-time PCR (qPCR), using TaqMan methodology, to measure the expression of MAGE-A1, A2, A3, A4, A6, A10, and A12. In addition, all 49 samples were tested by qPCR for expression of genes MAGE-C2, LAGE-2/NY-ESO-1 and GAGE. Quantification was obtained using standard curves, as explained in the Material and Methods-section and figure 1. Expression of GAGE was tested with a qPCR assay that detects GAGE-1, 2, and 8. CGG expression levels were calculated relative to β-actin expression (Fig. 2). For instance, the MAGE-A1/β-actin ratio for the first neuroblastoma sample was $1.1x10^{-2}$, indicating that 1.1 cDNA copies MAGE-A1 were present for every 100 cDNA copies of ß-actin.

Neuroblastoma. Eight of the 10 samples expressed at least 3 of the 7 MAGE-A genes that were tested. Expression of MAGE-C2, NY-ESO-1 or GAGE-1,2,8 was detected in 5 of the 8 MAGE-A-positive samples and in none of the negative samples. The pattern of expression of CGGs appeared to be highly clustered: all positive samples expressed at least 3 of the 10 genes tested (Table 2). The levels of CGG expression in the neuroblastoma samples were usually high. For example, 3 samples expressed gene MAGE-A10 at a level that was only 100-fold lower than that of the expression of the β-actin gene (Fig. 2). Four out of the 10 samples expressed at least 1 CGG at a CGG/β-actin ratio $>10^{-3}$.

Figure 2



Cancer-germline gene (CGG) expression in pediatric solid tumors measured by reverse transcription and quantitative real-time PCR. Each panel shows the results for one CGG in 49 different tumor samples (expression of GAGE was tested with a qPCR assay that detects *GAGE-1,2,* and *8*). Samples are arranged in the same order in all panels. The bars represent normalized CGG expression values (CGG/*ß-actin* ratios). Small circles indicate samples in which the distribution of the MAGE-A1, MAGE-A4 and NY-ESO-1 proteins was assessed by immunohistochemistry (see fig. 3 + 4).

Ewing's sarcoma. Eleven of the 18 Ewing's sarcoma samples expressed at least one CGG. Expression of MAGE-A1, MAGE-A2 and NY-ESO-1 could not be detected in any sample. Although a trend towards co-expression of multiple CGGs was observed, the number of co-expressed genes was less than in the other tumor types. The level of CGG expression in Ewing's sarcomas was always low (CGG/β-actin ratios $<1x10^{-3}$).

Rhabdomyosarcoma. Of the 12 samples, 6 expressed at least one CGG. The clustered pattern of expression of CGGs was most obvious in this tumor type: although 6 samples expressed no CGGs, 3 samples expressed at least 7 of the 10 genes. The level of expression in the positive samples was high. Three samples exhibited expression ratios $>10^{-3}$ for one or more CGGs. These 3 samples also expressed NY-ESO-1 at levels $>10^{-4}$.

Osteosarcoma. The highest incidence of CGG expression was observed in these tumors: all 9 samples expressed at least 4 of the 7 MAGE-A genes that were tested. All samples expressed MAGE-A3 and A6. Seven samples expressed MAGE-C2, 8 expressed NY-ESO-1, and 9 expressed GAGE-1,2,8. Each sample expressed at least 6 of the 10 CGGs. MAGE-A4 was expressed less frequently (4 positive samples) than the other CGGs (6-9 positive samples). Osteosarcoma samples also expressed CGGs levels: in 8 of the 9 samples the CGG/β-actin ratio of at least one CGG was higher than 10^{-3}. Expression ratios as high as 10^{-3} were observed for all CGGs except for MAGE-A4.

Table II. Clustered pattern of cancer-germline gene expression in neuroblastoma

Sample	MAGE A1	MAGE A2	MAGE A3	MAGE A4	MAGE A6	MAGE A10	MAGE A12	MAGE C2	NY-ESO-1	GAGE 1,2,8
1	++++	+++	++	+	+++	++++	+++	+++	+	++
2	+++	-	-	+++	++	++++	+	-	-	++
3	+++	+++	+++	-	+++	++	+++	+	++	+
4	++	++	++	+	+	++	++	+	+	-
5	++	++	++	-	++	++	++	-	-	-
6	++	++	+	-	+	-	+	-	-	-
7	-	+	++	+	+	-	++	+	+	+
8	-	-	-	+	++	++++	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-

The signs +, ++, +++, and ++++ represent CGG/ß-actin ratios ranging between 10^{-5} and 10^{-4}, 10^{-4} and 10^{-3}, 10^{-3} and 10^{-2}, and ratios > 10^{-2}, respectively (see fig. 2). The sign – indicates that no CGG expression could be detected.

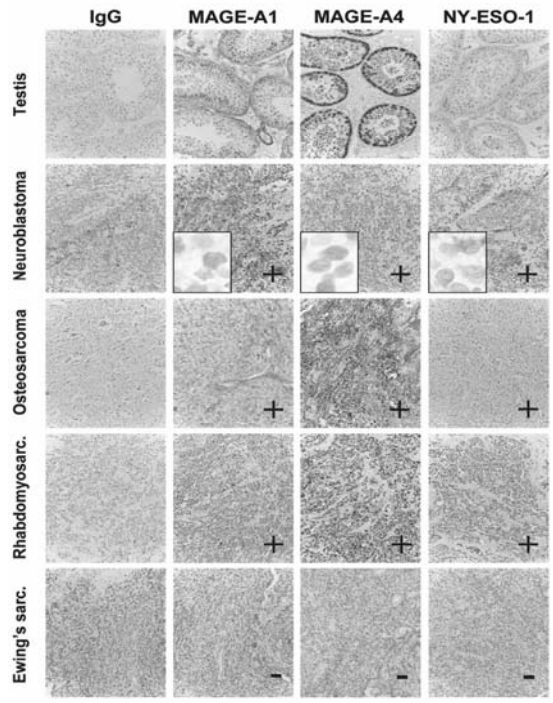
Cancer-germline protein distribution

Immunochemistry with monoclonal antibodies E978 (anti-NY-ESO-1), MA454 (anti-MAGE-A1) and 57B (anti-MAGE-A4) was performed on tissue sections from 9 tumors (identified with a circle in fig. 2). Sections from normal testis tissue were used as positive controls. Representative results are shown in fig. 3. In testis, the 3 antibodies labelled spermatogonia and spermatocytes; spermatozoa were not stained. The staining with antibody E978 was less intense compared to antibodies MA454 and 57B, both in testis and tumor tissues. In tumors, the staining with MA454 or 57B was homogeneously distributed throughout the tissue, except for one neuroblastoma

sample which displayed a heterogeneous staining pattern with all 3 antibodies. This is illustrated in fig. 3, with high magnification inserts showing positive tumor cells positioned next to negative tumor cells in sample neuroblastoma 1. In the other positive tumors, the staining of the tumor cells with E978 had an homogeneous (1 tumor), heterogeneous (1 tumor), or focal (1 tumor) distribution. Immunohistochemistry and qPCR data of the 9 tested tumors correlated well. Staining of the tumor cells with each of the three antibodies was observed only in sections from tumors that tested positive for mRNA expression. No false-negative or false-positive immunohistochemistry result was observed in any of the 27 sections that were analysed (chi-square, $p<0.001$). No staining was observed with the IgG isotype negative control antibody.

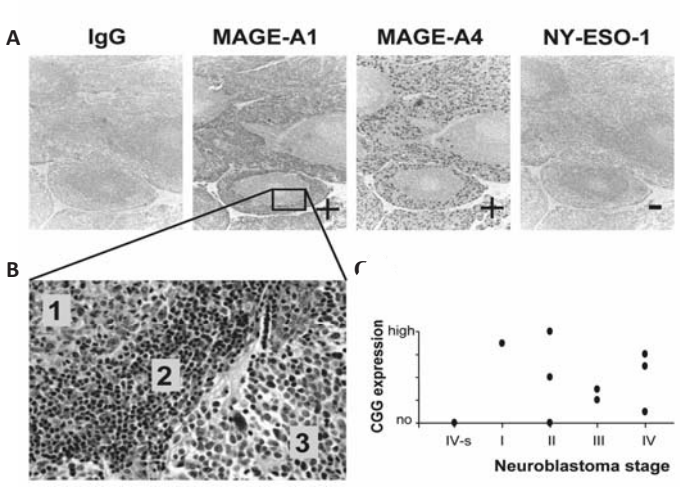
The immunostaining of MAGE-positive cells in sample neuroblastoma 2, a metastatic lymph node from a neuroblastoma patient, clearly showed that the tumor cells invaded the parafollicular areas of the node. The B cell areas, namely the follicle and the mantle zone, were not infiltrated by tumor cells (Fig. 4A). Hematoxylin-eosin staining confirmed that no tumor cells were present in the follicle and mantle zone (Fig. 4B).

Figure 3



Immunohistochemistry with monoclonal antibodies MA454 (anti-MAGE-A1), 57B (anti-MAGE-A4), and E978 (anti-NY-ESO-1), and with IgG isotype negative control antibody on sections of normal testis (positive control) and pediatric tumor tissues (samples neuroblastoma 1, osteosarcoma 2, rhabdomyosarcoma 3, and Ewing's sarcoma 8 in fig. 2). Original magnification 200x. The presence or absence of mRNA of *MAGE-A1, MAGE-A4* or *NY-ESO-1* in the sample (as tested by reverse transcription and qPCR; see fig. 2) is indicated by + or – for comparison. High magnification inserts (630x) demonstrate the heterogeneous staining patterns observed with all 3 antibodies in sample neuroblastoma sample 1.

Figure 4



Immunohistochemistry on tissue sections of metastatic neuroblastoma lymph-node sample 2 (A). Original magnification 100x. (B) Neuroblastoma sample 2, hematoxylin-eosin staining, original magnification 400x. Numbers in the figure represent 1. follicle; 2. mantle zone; 3. neuroblastoma tumor cells. (C) Correlation between cancer-germline gene (CGG) expression by the tumor and tumor stage in neuroblastoma. For each of the 10 tumors, CGG mRNA expression is reported as the sum of all CGG/*ß-actin* ratios shown in fig. 2. Neuroblastoma IV-s is a special type of neuroblastoma characterized by metastatic disease with spontaneous regression and good survival.[57] There is no significant correlation between level of CGG expression and neuroblastoma stage (r = 0.09; p = 0.79, calculated with the Spearman rank correlation).

CGG expression and tumor stage

Although the number of samples in each group of tumors was limited, the correlation between CGG expression and clinicopathological parameters was statistically analysed. In none of the four groups a significant correlation could be observed between CGG mRNA expression by the tumors and parameters such as tumor stage (Fig. 4C shows the results for neuroblastomas), age of the patient and clinical outcome (data not shown).

DISCUSSION

The identification of tumor-specific antigens is an essential step in the development of therapeutic cancer vaccines. The importance of antigens encoded by CGGs as vaccine targets has led to detailed studies of their expression in various tumors.[1,31] Little information is available regarding expression of CGGs in pediatric tumors. Here we report CGG expression in pediatric solid tumors using quantitative real-time PCR. We observed that all osteosarcoma, most neuroblastoma and some rhabdomyosarcoma tumors expressed several CGGs at high levels. Ewing's sarcoma samples had an overall low CGG expression. CGG expression was not correlated with tumor stage or clinical parameters of the patients. CGG protein expression was confirmed by immunohistochemistry, with staining patterns that were frequently homogeneous.

In immunotherapeutic trials with defined tumor antigens, the level of expression of the target antigens may be of great importance for the success of the vaccine. Real-time quantitative PCR is a sensitive method for quantifying gene transcripts and is as such preferable to semi-quantitative mRNA detection methods such as Northern blot and conventional PCR. Quantification of mRNA with real-time PCR needs appropriate standardization of the procedures for RNA extraction from tissues, cDNA synthesis, and the real-time PCR itself.[32] We had every tumor sample analysed by the pathologist and selected only samples with more than 80% tumor cells. In addition, we calculated the mRNA expression levels of CGGs relative to those of the reference gene *ß-actin*.

Our results indicate that CGG/*ß-actin* ratios as high as 0.1 can be observed in some pediatric tumor samples. High CGG expression (CGG/*ß-actin* ratio > $1x10^{-3}$) was observed in most neuroblastoma samples and in 100% of the osteosarcomas. In the Ewing's sarcomas and rhabdomyosarcomas we observed expression of at least 1 CGG in 61% and 50% of the samples, respectively. The CGG/*ß-actin* ratio in Ewing's sarcoma was lower than $1x10^{-3}$ in all samples. Non-quantitative data on the expression of *MAGE-A1*, *MAGE-A2*, *MAGE-A3* and *NY-ESO-1* in pediatric neuroblastoma and rhabdomyosarcoma have been reported by others.[33-38] These data and ours correlate well.

We frequently observed co-expression of multiple CGGs in our tumor samples. This phenomenon has been described also for other tumors, and is most likely a consequence of a global demethylation of the genome in the tumor.[39,40] It is also possible that activation of a single CGG leads to the activation of other CGGs (reviewed by Simson et al.).[31] The pattern of expression of CGGs that we observe in neuroblastoma is similar to that observed in melanomas,[41,42] which also develop from progenitor cells originating from the neural crest.

Numerous studies indicate that CGG expression is correlated with advanced pathologic stage and worse prognosis in various tumor types, including gastric carcinoma,[43,44] colorectal cancer,[45] breast cancer,[46,47] ovarian neoplasm,[48] bladder cancer,[49] non-small cell lung cancer[50,51] and multiple myeloma.[52,53] However, CGG expression is not correlated with disease progression in other malignancies such as neuroblastoma,[33-36] melanoma[54] and esophageal cancer.[55] We observed CGG expression in newly diagnosed tumors, in tumors collected after treatment with chemotherapy (osteosarcoma samples 5, 6 and 8 in fig. 2), and in metastases. We

could not detect a correlation between CGG expression in the tumor and clinicopathological parameters of the patients. Therefore, vaccination of these patients with CGG antigens is conceivable at all disease stages, including when a large tumor burden is present. And considering that pediatric tumors tend to co-express multiple CGGs, one may target several antigens in the same patient, which should reduce the risk of resistance through antigen-loss tumor variants.[56]

Proteins MAGE-A1, MAGE-A4 and NY-ESO-1 were visualized with immunohistochemistry. MAGE-A1 and MAGE-A4 were homogeneously present in most tumors. NY-ESO-1, in contrast, had homogeneous, heterogeneous or focal distribution patterns. The 27 immunohistochemical stainings of the 9 tested tumors correlated well with the qPCR data. For a complete overview of CGG expression in a tumor, PCR data are essential because for most CGGs no specific antibodies are available. It is of note that in neuroblastoma sample 2, no *MAGE-A3* mRNA expression was detected (Fig. 2) but staining of tumor cells by monoclonal antibody 57B was observed (Fig. 4A). This is in line with the observation that 57B, which was raised against a recombinant MAGE-A3 protein, does not detect MAGE-A3 but reliably detects MAGE-A4.[30] As shown in fig. 4A and B, the distribution of neuroblastoma cells in the lymph node of neuroblastoma sample 2 had a unique pattern: the B cell areas were free of tumor cells throughout the lymph node. We hypothesize that the microenvironment in the mantle zones of this lymph node prevents the tumor cells from entering the B cell areas.

In conclusion, we report high levels of CGG expression in a large fraction of pediatric solid tumors. These data indicate that antigens encoded by these CGGs can be used to vaccinate children with these tumors.

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Chapter 5

Selective cancer-germline gene expression in pediatric brain tumors

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Abstract

Cancer-germline genes (CGGs) code for immunogenic antigens that are present in various human tumors and can be targeted by immunotherapy. Their expression has been studied in a wide range of human tumors in adults. We measured the expression of 12 CGGs in pediatric brain tumors, to identify targets for therapeutic cancer vaccines. Real Time PCR was used to quantify the expression of genes *MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MAGE-C2, NY-ESO-1* and *GAGE-1,2,8* in 50 pediatric brain tumors of different histological subtypes. Protein expression was examined with immunohistochemistry. Fifty-five percent of the medulloblastomas (n=11), 86% of the ependymomas (n=7), 40% of the choroid plexus tumors (n=5) and 67% of astrocytic tumors (n=27) expressed one or more CGGs. Immunohistochemical analysis confirmed qPCR results. With exception of a minority of tumors, the overall level of CGG expression in pediatric brain tumors was low. We observed a high expression of at least one CGG in 32% of the samples. CGG-encoded antigens are therefore suitable targets in a very selected group of pediatric patients with a brain tumor. Interestingly, glioblastomas from adult patients expressed CGGs more often and at significantly higher levels compared to pediatric glioblastomas. This observation is in line with the notion that pediatric and adult glioblastomas develop along different genetic pathways.

Introduction

Despite major advances in the treatment of childhood cancer, cancer remains a common cause of death for children > 1 year of age.[1] Twenty-two percent of all pediatric cancers are CNS tumors. Pediatric patients with relapsed cancers of the CNS have a poor prognosis, and therefore novel therapies for these patients are urgently needed. Immunotherapy against brain tumors presents unique challenges since the brain is considered an immune privileged site. However, recent studies demonstrated that the immune cells have access to the brain in spite of the blood-brain barrier.[2,3] Within malignant brain tumors the blood-brain barrier is generally considered non-functional. Progress in our understanding of immune responses to CNS tumors have already led to novel clinical applications.[4,5] Most experience has been obtained with immunotherapeutic trials with dendritic cell vaccinations, as reviewed by De Vleeschouwer et al in 2006.[6]

Immunotherapy is an attractive therapeutic option for pediatric cancer patients because of its mild toxicity, and because the child's immune system is more potent and flexible compared to adults.[7,8] However, implementation of immunotherapy in pediatric oncology has been hampered by the lack of known tumor-specific antigens on pediatric tumors.

Cancer-germline genes (CGGs) are expressed in a wide range of human tumors and have a highly restricted expression pattern in normal tissues.[9,10] Antigens encoded by CGGs have been extensively studied because of their immunogenicity, tumor specificity, and their expression in a significant proportion of adult tumors of various histological types. Recently, we have shown that CGGs are expressed in a large percentage of pediatric extra-cranial solid tumors.[11]

The aim of this study was to analyze CGG expression in pediatric brain tumors. We report the results of a quantitative real-time PCR analysis of the expression of 12 CGGs in a panel of medulloblastomas, ependymomas, tumors of the choroid plexus and astrocytic tumors.

Material and methods

Tumor samples

Fresh-frozen tumor samples were available at the Department of Pathology at the Radboud University Nijmegen Medical Centre. All samples were from pediatric patients (0 to 19-years-old) with a brain tumor diagnosed at the Department of Pediatric Hemato-Oncology. Sections of the frozen samples were stained with hematoxylin-eosin and reviewed by the pathologist to verify tumor histology and to evaluate the percentage of tumor cells. Samples were only considered for study if the contents of tumor cells was ≥ 80%.

RNA isolation and cDNA synthesis

Total RNA was isolated with TriZol reagent (Invitrogen, Carlsbad, CA) and samples were treated with Deoxyribonuclease I (Invitrogen) according to the manufacturer's protocol. To generate cDNA, 1 µg DNase-treated RNA was reverse-transcribed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo(dT)

primer and 50 units SuperScript II, according to the manufacturer's protocol. After first-strand synthesis, samples were diluted to a final volume of 100 µl with water.

Conventional PCR

Duplex PCR amplification of β-actin and GAPDH transcripts was carried out in a 25-µl reaction volume containing 2.5 µl of cDNA, 1X PCR Buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 100 µM each dNTP, 0.4 µM each primer, and 0.625 units Taq DNA polymerase (TaKaRa, Shiga, Japan). β-actin primers were as described.[12] GAPDH primers (originally available from Clontech, Palo Alto, CA; kindly provided by Dr B. Lethé) were 5'-TgAAggTCggAgTCAACggATTTggT-3' (sense) and 5'-CATgTgggCCATgAggTCCACCAC-3' (antisense). Cycling was performed in a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) as follows: 94°C for 4 min, followed by 22 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. Cycling was concluded with a final extension step at 72°C for 15 min. PCR products were fractionated in 1.3% agarose gel and visualized by ethidium bromide fluorescence (β-actin, 626 bp; GAPDH, 983 bp). PCR amplification of MAGE-A transcripts was carried out with the primer pair designed by Zammatteo et al.[13] These primers derive from a consensus nucleotide sequence for the last exon of the 12 MAGE-A genes and give amplicons of ~539 bp. PCR conditions were as described above, except that PCR was performed for 30 cycles.

Quantitative real-time PCR

Expression of cancer-germline genes (CGGs) and of the reference gene β-actin, was measured by quantitative PCR, based on TaqMan methodology, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). PCR reactions were prepared with the qPCR Core Kit w/o dUTP reagents (Eurogentec, Seraing, Belgium). Each reaction (25 µl) contained 2.5 µl of cDNA, 1X PCR buffer containing the passive reference dye ROX, 5 mM $MgCl_2$, 200 µM each dNTP, 200 nM each primer, 100 nM probe, and 0.625 units DNA polymerase. Primers, probes and thermal cycling conditions are given in table 1.[14,15] Probes with 6FAM and TAMRA labels were from Eurogentec. Probes with 6FAM and MGB-NFQ labels (for MAGE-A3, MAGE-A6 and MAGE-A12) were from Applied Biosystems. Quantification of the samples was achieved by extrapolation from a standard curve of serial dilution points of cDNA of the relevant gene. Samples and standard dilution points were assayed in duplicate or triplicate. Standard calibration curves for β-actin and all CGGs were linear over 4 (CGGs) or 5 (ß-actin) orders of magnitude and had similar PCR efficiencies (slope from -3.45 to -3.77). Differences in sensitivity between the assays for the various genes (y-intercept from 38.7 to 41.6) were due in part to differences in the actual cDNA copy number in the standard dilutions. cDNA copy numbers in the standards were verified by testing minimally 12 replicates of the 1-copy dilution in each qPCR run. If needed, copy numbers of the test samples were corrected by a factor calculated on the basis of the results for the 1-copy dilution. Normalization of samples was achieved by dividing the copy number of CGG by that of the reference gene, β-actin.

Table 1. Primers, probes and thermal cycling conditions of qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Probe (5'→3')	Annealing-extension
MAGEA1 [15]	gCC gAA ggA ACC TgA CC	ACT ggg TTg CCT CTg TCg	TgT gTg CAg gCT gCC ACC TCC T	90 s, 65°C
MAGEA2 [15]	AAg TAg gAC CCg Agg CAC Tg	gAA gAg gAA gAA gCg gTC Tg	CAT TgA Agg AgA AgA TCT gCC TgT ggg TCT TC	1 min, 60°C
MAGEA3 [14]	gTC gTC ggA AAT Tgg CAg TAT	gCA ggT ggC AAA gAT gTA CAA	AAA gCT TCC AgT TCC TT	1 min, 62°C
MAGEA4 [15]	CCA CTA CCA TCA gCT TCA CTT gC	CTT CTC ggA ACA Agg ACT CTg C	Agg CAA CCC AAT gAg ggT TCC AgC	1 min, 63°C
MAGEA6	gTC gTC ggA AAT Tgg CAg T	gCA ggT ggC AAA gAT gTA CAC	TgC AAg gAA TCg gAA gC	1 min, 65°C
MAGEA10	TAC TgC ACC CCT gAg gAg gTC	TgT ggT ggC AAT TCT gTC CTg	AAA Tgg gAg TgA TCC AAg ATC CTT CCC AC	1 min, 64°C
MAGEA12	ggT ggA AgT ggT CCg CAT Cg	gCC CTC CAC TgA TCT TTA gCA A	Agg CAT CTg ATg ggA gg	1 min, 60°C
MAGEC2	ggg AAT CTg ACg gAT Cgg A	ggA ATg gAA CgC CTg gAA C	TgC TCC TgA AgA AgT CgT CAT gCC TCC	1 min, 64°C
GAGE1,2,8 [a]	CTA gAC CAA gAC gCT ACg TAg A	CCC ATC Agg ACC ATC TTC ACA	CCT ATg Cgg CCC gAg CAg TTC Ag	1 min, 62°C
LAGE2/NY-ESO-1 [15]	CgC CTg CTT gAg TTC TAC C	CAC TgC gTg ATC CAC ATC AAC A	TCA gTA TgT TgC Cgg ACA CAg TgA ACT C	1 min, 62°C
ACTB [14]	ATT gCC gAC Agg ATg CAg AA	gTC ATA CTC CTg CTT gCT gA	TCA AgA TCA TTg CTC CTC CTg AgC gC	1 min, 60°C

[a] This assay detects GAGE1, GAGE2 and GAGE8.

Immunohistochemistry

Immunochemistry was performed on 4 μm tissue sections of formalin-fixed paraffin-embedded tissue blocks. Sections were heated for 20 min in citrate buffer (10 mM, pH 6.0) for antigen retrieval. The following mouse IgG1 monoclonal antibodies (mAb) were used: E978 (anti-*NY-ESO-1*)[16] (Zymed, San Francisco, CA), MA454 (anti-*MAGE-A1*)[17] (Zymed, San Francisco, CA), and 57B (anti-*MAGE-A4*)[18,19] (kindly provided by Dr. G.C. Spagnoli, University Hospital Basel, Switzerland). Testis tissue with intact spermatogenesis was used as positive control. Tissue sections were incubated with mAb diluted in PBA: E978 (2.5 μg/ml), MA454 (1 μg/ml), or 57B (5 μg/ml), or with IgG1 negative control antibody, at room temperature for 1 hour. Binding sites of primary antibodies were then detected by a biotinylated horse–antimouse secondary reagent (Vector Laboratories, Burlingame, CA) followed by an avidin–biotin complex system (ABC Elite, Vector Laboratories). Diaminobenzidine tetrachloride served as a chromogen. Immunoreactivity was assessed blindly with respect to the mAb used.

Statistical analysis

Normalized CGG values are presented as means ± standard deviation (SD). The SD of the normalized CGG values was calculated from the SD of the CGG and the β-actin values using the following formula: CV = SQRT [$CV^2_{\beta\text{-actin}} + CV^2_{CGG}$], where CV = SD/mean value (as described in the Sequence Detection System User Bulletin 2, 1997, Applied Biosystems). Differences in mRNA expression levels between pediatric and adult glioblastomas are calculated with the Spearman rank correlation. All statistical tests were two-sided, significance was determined as $p<0.05$.

Results

Study population

We analyzed cancer-germline gene (CGG) expression in 50 fresh-frozen tumors by reverse transcription and polymerase-chain-reaction (PCR) amplification. All samples were histologically proven brain tumors from pediatric patients, ≤ 19 years old at the time of tumor resection and classified according to the WHO 2007 classification[20] (Table 2). An extra-cohort of 9 glioblastoma samples from adult patients was also analyzed, for comparison to pediatric glioblastoma. Integrity of cDNA samples was verified by conventional, 22-cycle PCR amplification of a 626 bp *β-actin* and a 983 bp *GAPDH* product (data not shown). Samples were subjected to conventional PCR amplification with consensus primers for the 12 genes of the *MAGE-A* family. Twenty-seven samples were positive, indicating that, depending on the tumor type, 33 to 64% of the investigated pediatric tumors expressed at least one *MAGE-A* gene (Table 2).

Table 2. Study group

Tumor type[a]	**Number of patients**	**Average age (range)**	**% of *MAGE-A*-positive tumors**[b]
Medulloblastoma	11	12 years (4-19)	55
Ependymoma	7	7 years (1-12)	43
Choroid plexus tumor	5	4 years (1-10)	40
Pilocytic astrocytoma (WHO grade I)	14	6 years (3-15)	64
Diffuse astrocytoma (WHO grade II)	3	10 years (5-18)	33
Anaplastic astrocytoma (WHO grade III)	5	13 (7-19)	60
Glioblastoma (WHO grade IV)	5	13 (3-19)	60
Glioblastoma (WHO grade IV)[c]	9	59 (31-74)	100

[a] According to WHO 2007 classification[20]

[b] Gene expression was determined by conventional PCR with consensus primers for the 12 genes of the *MAGE-A* family.

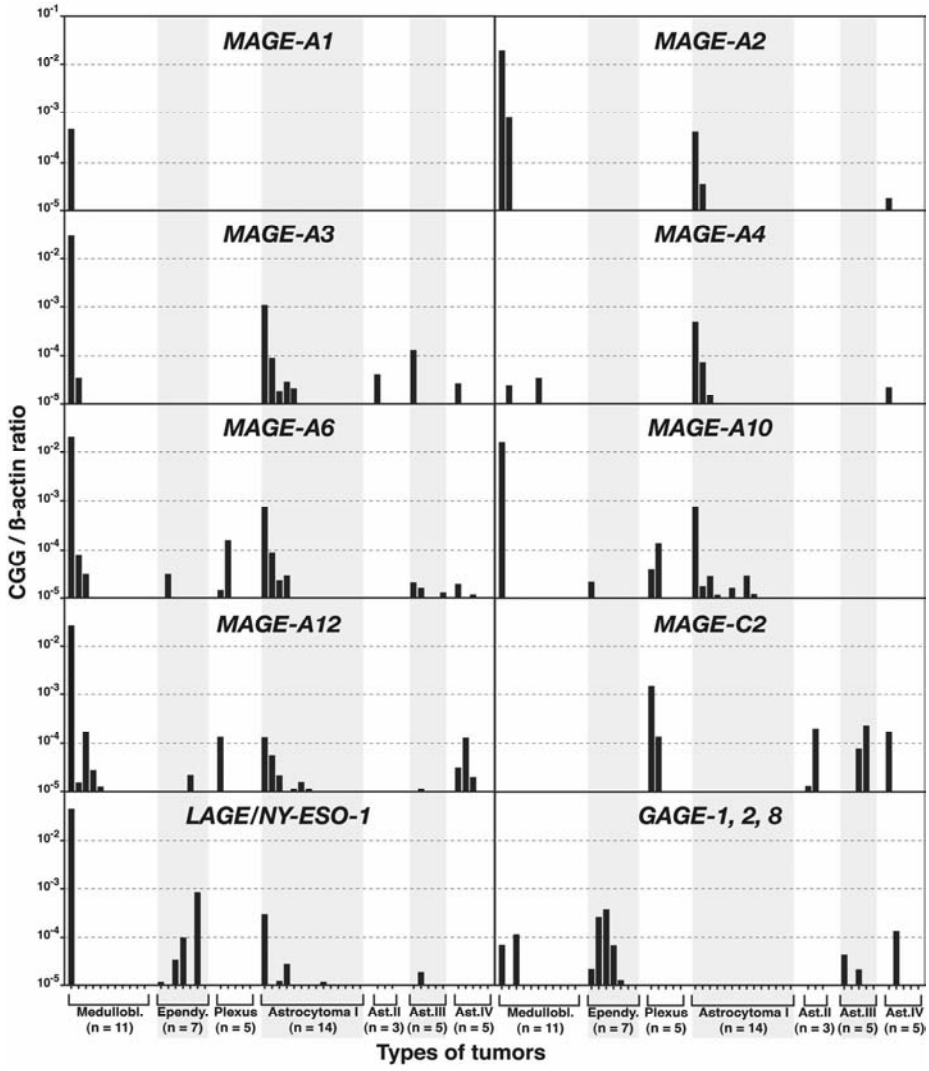
[c] An extra cohort of adult patients with glioblastomas was studied to compare to pediatric glioblastomas (figure 2).

CGG expression in pediatric brain tumors

The 27 *MAGE-A*-positive samples were subsequently analyzed by quantitative real-time PCR (qPCR), using the TaqMan methodology, to measure the expression of the individual MAGE genes, *MAGE-A1*, *A2*, *A3*, *A4*, *A6*, *A10*, and *A12*. In addition, all 50 samples were tested by qPCR for expression of genes *MAGE-C2*, *NY-ESO-1* and *GAGE-1,2,8* (table 1). CGG expression levels were normalized to those of the *β-actin* gene (figure 1).

Medulloblastoma. Six of the 11 medulloblastoma tumors expressed at least 1 of the 10 CGGs that were analyzed. The expression of CGGs in medulloblastomas was highly clustered (Table 3). One sample expressed high levels (CGG/*ß-actin* ratios $>10^{-2}$) of 6 CGG genes. The other medulloblastoma samples only sporadically expressed CGGs, with CGG/*ß-actin* ratios above 10^{-4}.

Figure 1



Cancer-germline gene (CGG) expression in pediatric brain tumors measured by reverse transcription and quantitative real-time PCR. Each graph shows the results for one CGG in 50 different tumor samples. Samples are arranged in the same order in all graphs. The horizontal axis indicates the tumor type (Medullobl., medulloblastoma; Ependy., ependymoma; Plexus, plexus choroideus; Ast., astrocytic tumors grade I to IV). The bars represent normalized CGG expression values (CGG/*ß-actin* ratios).

Ependymoma. Six out of seven ependymoma samples expressed at least 1 CGG. None expressed more than 3. Here the expression was therefore not clustered. Most positive samples expressed *NY-ESO-1* and/or *GAGE-1,2,8.* The overall levels of CGG expression in ependymoma samples were low, with CGG/*β-actin* ratios below $1x10^{-3}$.

Choroid plexus tumor. Of the 5 choroid plexus tumors analyzed, 3 showed no detectable CGG expression. Samples #1 and #2 expressed at least three CGGs. Only in one sample the CGG/*β-actin* ratio exceeded $1x10^{-3}$.

Pilocytic astrocytoma. Nine out of 14 pilocytic astrocytomas expressed at least one CGG. The overall expression was low, one low pilocytic astrocytoma had a CGG/*β-actin* ratio $>1x10^{-4}$.

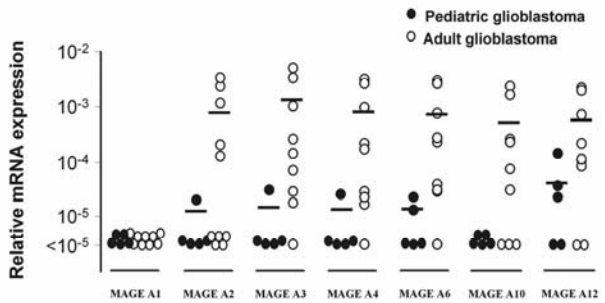
Diffuse astrocytoma. Two out of 3 diffuse astrocytomas expressed at least one CGG. The overall expression level of CGGs on diffuse astrocytomas was low.

High grade astrocytoma. Eight out of 10 anaplastic astrocytomas and glioblastomas expressed at least one CGG. CGG/*β-actin* ratio in high grade astrocytomas did not exceed $1x10^{-3}$.

CGG expression in pediatric vs adult glioblastomas

The relatively low expression of MAGE genes in high grade astrocytomas was unexpected since *MAGE* expression has been reported in almost all adult glioblastomas.[21-24] In these studies different methods were used, immunohistochemistry[21] and conventional, non-quantitative PCR.[22-24] Therefore we decided to select 9 new adult glioblastoma samples for *MAGE* qPCR analysis. All samples expressed at least 5 *MAGE-A* genes at high levels. The difference between the levels of *MAGE-A* genes expression in pediatric versus adult glioblastomas is shown in Figure 2 ($p=6.1x10^{-6}$).

Figure 2



MAGE-A expression in glioblastomas. Normalized MAGE-A expression levels (MAGE-A/*β-actin* ratios) of seven MAGE-A genes are shown. Horizontal bars represent mean relative MAGE expression. Pediatric glioblastomas (closed circles) express significantly lower levels of MAGE-A compared to adult glioblastomas (open circles), $p=6.1x10^{-6}$ calculated with Spearman rank correlation.

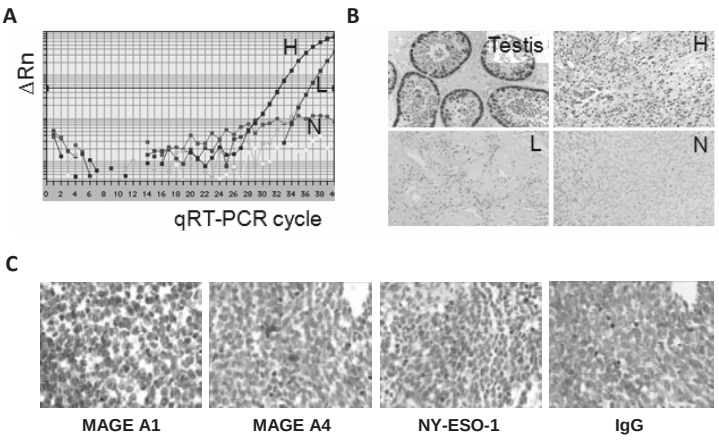
Cancer-germline protein distribution

Immunohistochemistry with monoclonal antibodies (mAbs) E978 (anti-NY-ESO-1), MA454 (anti-MAGE-A1) and 57B (anti-MAGE-A4) was performed on available paraffin-embedded tissues. Sections from normal testis tissue were used as positive controls. The intensity of the stainings correlated well with the level of CGG expression, as shown in figure 3a,b. The MAGE-A1, MAGE-A4 and NY-ESO-1 proteins were distributed homogenously throughout the tissues. However, a minority of the tissues had a heterogeneous staining pattern such as the MAGE-A4 expression in medulloblastoma sample #1 (figure 3c). No staining could be observed for samples that scored either negative or with CGG/*β-actin* ratios below 10^{-4}.

CGG expression and tumor stage

The number of patients in each specific group of tumors was too small to try to establish a correlation between CGG expression and clinicopathological parameters. Stratification of the pediatric astrocytic tumors in pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II) and high grade anaplastic astrocytomas and glioblastomas (grade III and IV, respectively), revealed that the level of CGG expression was not significantly different between groups. These data suggest that CGG expression in astrocytic tumors is not correlated with the grade of the astrocytic tumor in pediatric patients.

Figure 3



A) Amplification plots of three pilocytic astrocytomas in duplicate showing high numbers (H), low numbers (L) or no (N) MAGE-A4 cDNA copies. B) Immunohistochemistry with mAb 57B (anti-MAGE-A4) of the pilocytic astrocytomas shown in figure 3a. A section of normal testis is used as a positive control. The intensity of the staining correlates with the amount of MAGE-A4 copies. C) Immunohistochemistry with mAbs E978 (anti-*NY-ESO-1*), MA454 (anti-*MAGE-A1*), 57B (anti-*MAGE-A4*) and the IgG isotype negative control antibody on sections of medulloblastoma sample 1 (see figure 1 for relative mRNA expression). Original magnification 63x. This sample was chosen because of the heterogeneous expression of the MAGE-A4 protein.

Discussion

Antibodies and T cells can be identified that recognize antigenic fragments derived from gene products expressed by tumors.[10,25,26]. A critical role for these anti-tumor immune mechanisms in the eradication of cancer has been demonstrated in numerous animal models and some clinical trials.[27,28] In the last decennia promising new strategies for the development of antibodies and activated T cells against tumor associated antigens have been developed.[29,30] In spite of the blood brain barrier, there is accumulating evidence that even brain tumors can cause immune activation and are amenable for immunotherapy.[2,4,31]

The identification of immunogenic tumor associated antigens is an essential step in the development of rational cancer vaccines. The potential of CGGs as vaccine targets has led to detailed studies of their expression in various malignancies in adult patients.[10,32] Previously, we reported on the expression of CGGs in pediatric extra-cranial tumors.[11] Here we report that, in our cohort of 50 pediatric brain tumors, 68% expressed one or more CGGs. Immunohistochemical data correlated well with the qPCR results. Apart from a few exceptions, the overall level of CGG expression on pediatric brain tumors is low. Preliminary data from cytotoxicity assays indicated that glioblastoma cell lines that express low levels of CGGs (CGG/*β-actin* ratio < $1x10^{-4}$) are not specifically lysed by HLA-matched anti-MAGE A3 CTL cell lines (data not shown).

In contrast to the limited CGG expression found in pediatric brain tumors, glioblastomas from adult patients express significantly higher levels of CGGs ($p<0,001$). This finding is in line with reported differences between adult and pediatric glioblastomas in p53 and EGFR expression.[33,34] The differences in protein expression may be explained other chromosomal aberrations and differences in microsatellite stability between adult glioblastomas and pediatric glioblastomas.[35,36] The significant difference in MAGE-A expression between adult and pediatric glioblastomas supports the view that these tumors develop along distinct genetic pathways.

In conclusion, we report limited CGG expression in pediatric brain tumors. Only a small percentage of brain tumors express high levels of CGGs. These data indicate that CGGs can only be used as immune target in a selected group of pediatric brain tumors.

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Chapter 6

Vaccine-specific T cell reactivity in immunotherapy-associated vitiligo in melanoma patients

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Abstract

The occurrence of vitiligo in patients with melanoma is especially reported for patients undergoing immunotherapy. While vitiligo in these patients is thought to be related to an immune response directed against melanoma cells, solid evidence is lacking.

Here we report local cytotoxic T cell reactivity in three melanoma patients who developed vitiligo, after experimental immunotherapy using dendritic cell vaccinations. Tetramer analysis showed that vaccine-induced T cells recognizing gp100 and tyrosinase are present at the vitiligo lesions. These T cells secrete IFN-γ and IL-2 upon peptide specific stimulation as well as upon recognition of the autologous tumor.

We show that functional $CD8^+$ T cells specific for melanoma differentiation antigens used in a melanoma immunotherapy trial, do not only invade the tumor, but also the vitiligo lesions. This directly links vitiligo to the immuno-therapeutic intervention and supports the hypothesis that vitiligo is a marker of immunity against melanoma cells.

Introduction

Vitiligo is a common skin disease that is characterized by depigmented lesions associated with local destruction of melanocytes.[1] For melanoma patients the chance to develop vitiligo is estimated to be seven to ten-fold higher compared to the general population,[2] especially when they participate in an immunotherapeutic trial.[3] Based on numerous observations, development of *de novo* vitiligo lesions in melanoma patients is generally regarded as a favourable prognostic factor.

The etiology of vitiligo is not yet completely understood, but several lines of evidence suggest that both spontaneous and immunotherapy-associated vitiligo are immune-mediated processes. In spontaneous vitiligo, circulating anti-melanocytic antibodies and lymphocyte infiltrations at the margins of progressive lesions are present.[4,5] Furthermore, spontaneous vitiligo is correlated with circulating cytotoxic T lymphocytes (CTLs) specific for melanoma differentiation antigens (MDAs).[6] Analysis of vitiligo infiltrating lymphocytes (VILs) isolated from vitiligo lesions showed a local enrichment of predominantly Melan-A specific T cells.[7-9]

Numerous melanoma antigens are shared by normal melanocytes; amongst these are MDAs such as Melan-A/MART-1, gp100, tyrosinase and tyrosinase related proteins.[10,11] In two murine melanoma models, it is shown that *de novo* development of vitiligo is associated with reduced tumor progression. In both models, MDA-specific CTLs play a crucial role in both tumor control and melanoma-associated vitiligo.[12,13] Also in humans the appearance of vitiligo during the course of melanoma is thought to be caused by a cross-reactive immune response directed against melanoma cells, which explains the better prognosis in these patients reported in literature.[14,15]

Here we describe three melanoma patients who developed vitiligo after immunotherapeutic intervention. Patients were vaccinated with autologous monocyte-derived dendritic cells (DCs) loaded with gp100 and tyrosinase epitopes.[16] We demonstrate that CTLs against these epitopes can be detected in low percentages in the peripheral blood after vaccination. Interestingly, functional CTLs with the same specificity were observed in high percentages in both tumor and vitiligo lesions, which supports the hypothesis that the vitiligo is a direct cross-reactive effect of the anti-melanoma immunotherapy.

Material and methods

Patient selection

Melanoma patients with regional lymph node and distant metastases, participated in an experimental study using autologous monocyte-derived DCs loaded with HLA-A2.1 compatible tumor antigens gp100 and tyrosinase.[16] Only patients who developed vitiligo after immunotherapy, clinically confirmed by a dermatologist, were selected. Blood samples and 6 mm biopsies of perilesional vitiligo were obtained after informed consent. Approval from the local regulatory committee was obtained.

Preparation of vitiligo-infiltrating lymphocytes (VILs).

Six mm punch biopsies of the perilesional vitiligo-site (figure 1a) were obtained and cut in half. One part was cryopreserved for immunohistochemistry at a later timepoint, the other part was disrupted and a cell suspension was made by gentle squeezing in a sterile open filter chamber (NPBI, Amsterdam, Netherlands) in RPMI 1640/7% human serum (Sanquin, Nijmegen, Netherlands) supplemented with IL-2 (100 U/ml). The cell suspension was plated in a 24-wells plate (Costar Badhoevedorp, The Netherlands). T cells were morphologically and functionally tested after 2 weeks of culture.

Preparation of tumor-infiltrating lymphocytes (TILs) and melanoma cell lines.

TILs were obtained and cultured according to the same protocol as the vitiligo cell suspension (see above). A tumor cell line was generated by tumor culture in DMEM (Gibco - Invitrogen) supplemented with 10% fetal calf serum (Greiner). Fresh medium was added twice a week.

MHC Tetramer staining.

VILs and TILs ($50x10^3$ cells in 10 µl) or $1x10^6$ peripheral blood mononuclear cells in 10 µl, were incubated with directly labeled $gp100^{154-162}$, $gp100^{280-288}$, $tyrosinase^{369-376}$, $Melan\text{-}A^{26-35}$ and HIV^{77-85} tetrameric-MHC-HLA-A2.1 complexes for 60 minutes at room temperature. FITC-conjugated monoclonal antibodies directed against CD8 (Becton Dickinson) were added during the last 20 minutes. After washing, the samples were analyzed by flow cytometry. Percentage tetramer positive cells were calculated over the $CD8^+$ fraction. All samples were tested with HIV^{77-85}-HLA-A2.1-tetramers recognizing the irrelevant HIV-peptide SLYNTVATL as background control.[16] Control tetramer binding was less than 0.02% in all samples (data now shown).

Cytokine production.

Production of cytokines by VILs and TILs were measured in response to specific stimuli (target:effector-ratio 1:1). Two different target cells were used: T2 cells pulsed with $gp100^{154-162}$, $gp100^{280-288}$, $tyrosinase^{369-376}$ or the irrelevant peptide HIV^{77-85} and BLM cells (a melanoma cell line expressing HLA-A2.1 and no endogenous expression of gp100 and tyrosinase) transfected with control antigen G250, gp100 or tyrosinase. IFN-γ and IL-2 production were measured in supernatants after 16 hours by cytometric bead array according to the manufacturers protocol (Th1/Th2 Cytokine CBA 1; BD Pharmingen).

Antibodies and immunostaining.
Regulatory T cell phenotype was assessed by flowcytometry using mAbs directly conjugated to fluorescent dyes. The FOXP3-FITC (clone PCH101, eBioscience, San Diago, CA), CD4-APC, CD25-PE, CD127-PE-Cy5 (all Becton Dickinson) were used according to manufacturers protocols. Regulatory T cells were defined as $CD4^{+}FoxP3^{+}CD25^{+}CD127^{-}$. For immunohistochemistry, the following mAbs were used: M2-7C10+M2-9E3 (Labvision, Fremont, CA) against Melan-A, SP7 (Labvision) against CD3, HMB-45 (Dako, Glostrup, Denmark) against gp100 and T311 (Novocastra, Newcastle, United Kingdom) against tyrosinase.[17]

Results and Discussion

Patients

We prospectively followed three HLA-A2.1 positive patients with histologically documented melanoma who received DC-based immunotherapy and subsequently developed vitiligo (table 1).

Patient 1. Patient 1 was diagnosed, two years prior to inclusion, with melanoma of the scalp with regional lymph node metastases (T3aN2aM0), for which a radical lymph node dissection was performed. She relapsed with regional lymph node and cutaneous metastases, which were resected and treated with adjuvant radiotherapy. At inclusion, this patient presented with multiple subcutaneous and lung metastases. The patient was vaccinated intranodally with antigen loaded mature DCs[16] but treatment was discontinued after 3 vaccinations because of progression of cutaneous metastases. She developed vitiligo in the flank during further tumor progression.

Patient 2. Patient 2 was diagnosed, twelve years prior to inclusion, with melanoma located on the right lower leg (T1aN0M0), which was surgically removed. From 1997 on, the patient relapsed with in transit and distant cutaneous metastases, for which he was treated with regional limb perfusion (melfalan) and dacarbazine. At inclusion, he was diagnosed with lymph node metastases and multiple (sub-)cutaneous metastases for which he was repeatedly vaccinated intranodally with antigen loaded mature DCs. After 3 vaccinations, the patient developed vitiligo in the neck region and dorsal side of both hands. To date, the vitiligo still progresses and affects the head, back, hands and legs.

Patient 3. Patient 3 was diagnosed, 6 months prior to inclusion, with a superficial spreading melanoma of the left thigh (T1a/bN0M0). At inclusion she presented with an inguinal lymph node metastasis, for which a radical lymph node dissection was performed. She was vaccinated intradermally in an adjuvant setting with antigen loaded mature DCs. After 8 vaccinations, she developed a severe rash in the neck region, thorax, back and upper extremities and subsequent vitiligo in these areas.

Vitiligo development in melanoma patients upon vaccination with DCs has been reported before.[18,19] Although not investigated, it is currently believed that the vitiligo in these patients results from sensitization to antigens shared by melanocytes and melanoma cells. To address this question we took perilesional biopsies and studied the vitiligo infiltrating immune cells.

Vaccine specific lymphocytes infiltrate vitiligo lesions

Immunohistochemical analysis of the vitiligo in all three patients demonstrates perilesional infiltration of T cells (figure 1a,b). With tetramer analysis, $CD8^+$ T cells specific for one or more gp100 and tyrosinase HLA-A2.1 epitopes were detected at low frequencies in the peripheral blood after vaccination in all three patients. Gp100 or tyrosinase specific $CD8^+$ T cells were not detectable prior to vaccination (data not shown). Interestingly, specific CTLs were detected at increased concentrations in the vitiligo lesion compared to peripheral blood (table 1). This phenomenon was most obvious in patient 3 (figure 1c,d). The accumulation of these CTLs in the vitiligo lesions

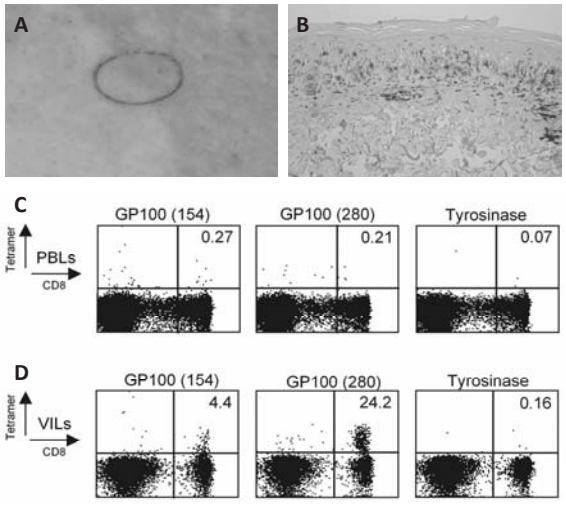
Table 1. Patient characteristics and tetramer specific CTLs after immunotherapy

Pt / sex / age (yrs)	TNM (stage)	Localisation	Prior treatment	Vacci-nations [a]	TTP	Current status (months)		Tetramer specific CTLs after immunotherapy [b]			
								gp100^{154} epitope	gp100^{280} epitope	Tyro-sinase	Melan-A
1. / F / 20	M1b (IV)	Lung, skin, LN	Post-surgery RTx	3	7	AWD (21)	DTH	n.a.	n.a.	n.a.	n.a.
							Blood [c]	0.01	0.03	0.00	0.03
							Vitiligo	0.05	0.02	0.00	1.4
							Melanoma	0.32 [d]	0.04	0.02	1.7 [d]
2. / M / 71	M1a (IV)	Distant skin	Dacarbazine, Melfalan, DNCB cream	3	38	AWD (56)	DTH	0.06	0.04	0.15	n.a.
							Blood [c]	0.10	0.82	0.01	0.01
							Vitiligo	0.00	1.6	0.77	1.13
3. /F / 53	N1b (III)	LN	none	7	NDD	NDD (21)	DTH	0.01	0.04	0.01	n.a.
							Blood [c]	0.27	0.21	0.07	0.06
							Vitiligo	4.40	24.2	0.16	0.05

[a] number of vaccinations prior to occurrence of vitiligo
[b] Percentage of tetramer specific CD8+ T cells compared to total CD8+ T cells
[c] no specific CTLs for gp100, tyrosinase and Melan-A were detected in the blood prior to immunotherapy
[d] flowcytometry results for gp100 specific CTLs infiltrating the melanoma are shown in figure 3a,b
LN = lymphnode, TTP = time to progression (months), AWD = Alive with disease, NDD = no detectable disease, DTH = delayed type hypersensitivity-assay, n.a. = not available

can result from specific migration[20] and/or a local proliferation advantage for MDA specific CTLs induced by perilesional antigen exposure.
Although the immunotherapy was not primarily directed against the Melan-A epitope, Melan-A specific CTLs were observed in the vitiligo lesions and tumor lesion of patient 1 and 2 (table 1 and figure 3). This finding is consistent with the observation that anti-tumor vaccines can have effects beyond their intrinsic specificity. It is shown that the interaction of vaccine specific T cells with melanoma cells may trigger a broad activation of other anti-tumor T cells, a phenomenon called epitope spreading.[21,22]

Figure 1



Vaccine-specific CTLs accumulate at the vitiligo lesion. Perilesional 6-mm skin biopsies were taken from each patient (A). CD-3 staining of the periolesional skin showing lymphocyte infiltrates (B), original magnification 200x. Flowcytometry of peripheral blood lymphocytes (PBLs, C) and vitiligo infiltrating lymphocytes (VILs, D) from one representative patient (patient 3). From the scatterplot the lymphocytes were gated and double stained with anti-CD8-FITC and tetramer-PE. The numbers in the dot plots indicate the percentage of tetramer-reactive cells of the CD8$^+$ cell fraction.

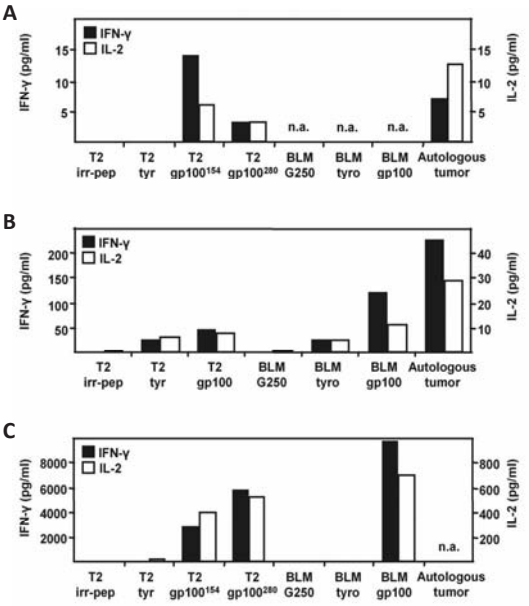
Vitiligo infiltrating lymphocytes produce IFN-γ after specific stimulation

To test whether vitiligo infiltrating lymphocytes (VILs) were functional, we stimulated these cells with gp100 or tyrosinase expressing target cells and measured subsequent IFN-γ and IL-2 production. VILs of patients 1 and 3 produce IFN-γ and IL-2 when exposed to gp100-target cells and VILs of patient 2 produce IFN-γ and IL-2 when exposed to both gp100 and tyrosinase-target cells. No cytokines were produced when VILs were exposed to unloaded control target cells (figure 2).

From patients 1 and 2 we were able to culture melanoma cell lines from metastases. Interestingly, the VILs also produced IFN-γ and IL-2 when stimulated by the autologous tumor cell line (figure 2). The cross reactivity of the VILs with autologous tumor cells further supports the hypothesis that the vitiligo is a marker of immunity against melanoma cells.

Vitiligo lesions in our patients did not specifically occur at the vaccine injection-site, at the site of the primary tumor, or in close proximity of new metastases. From this we conclude that the melanoma-associated vitiligo is not caused by a direct and local side-effect of tumor destruction, but rather by the presence of circulating MDA-specific CTLs. Although we did not use MHC class II peptides in our vaccination protocol, our data do not exclude that specific $CD4^+$ cells are involved. MDA-specific epitopes recognized by $CD4^+$ T cells have been reported[23] and it is also known that melanocytes can present peptides in an MHC class II restricted manner.[24] These observations suggest that melanocyte destruction may not only depend on MHC class I-restricted cells.

Figure 2



Vitiligo infiltrating lymphocytes produce IFN-γ and IL-2 upon specific stimulation. IFN-γ (black bars) and IL-2 (white bars) production of VILs after stimulation with non-specific and specific stimuli of patient 1 (A) patient 2 (B) and patient 3 (C). T2 cells are loaded with respectively irrelevant peptide, tyrosinase peptide and gp100 peptides. BLM cells are transfected with respectively control protein G250, tyrosinase protein and gp100 protein (see also material en methods). From patients 1 and 2 melanoma cell lines were cultured, the VILs produced high levels of IFN-γ and IL-2 when stimulated by the autologous tumor cells. N.a. not available.

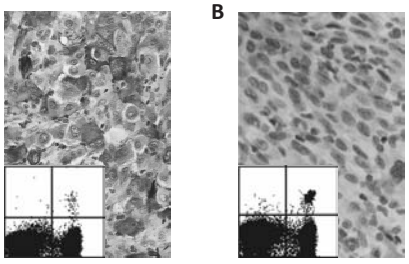
Immunotherapy-associated vitiligo and prognosis

Our case series is too limited to afford decisive conclusions on the prognostic value of vitiligo on melanoma disease. The presence of durable non-progressive disease in patients 2 and 3 suggest that the immunotherapy induced vitiligo lesions are a favourable prognostic factor. We further show that all three patients have a relatively high number of MDA-specific T cells in the blood, DTH biopsies and melanoma. Earlier papers have described a correlation with the presence of MDA-specific T cells in these compartments and favourable outcome.[16,25]

Despite the presence of functional melanoma specific T cells, patient 1 had progressive disease. This is intriguing since the tumor expressed the MDAs gp100 and Melan-A (IHC figure 3a,b) and was infiltrated by CTLs that specifically recognize these epitopes (inserts figure 3a,b and table 1). Several mechanisms may account for the escape of melanoma cells to immune surveillance.[26] It has recently been published that $CD4^+FoxP3^+CD25^+$ regulatory T cells (Tregs) can infiltrate melanoma tumors and locally suppress cytotoxic anti-tumor responses, also in vaccinated melanoma patients.[27] Infiltration of melanoma tumors with Tregs correlates with poor prognosis.[28] In the melanoma metastasis of patient 1, we detected that 27% of the tumor infiltrating $CD4^+$ T cells consisted of $CD4^+FoxP3^+CD25^+CD127^-$ Tregs vs 4% of Tregs in the blood of this patient (data not shown).

Altogether, our data support an association between vitiligo and favourable outcome. However, active vaccine-induced vitiligo does not exclude tumor progression. The dichotomy between MDA-specific CTL responses in melanoma and vitiligo might result from quantitative CTL differences, the quality of the CTLs such as T cell receptor affinity and cytokine production,[29] the amount of antigen presented[30,31] and the different environmental conditions in which these T cells exert their function.[26]

Figure 3



Accumulation of MDA-specific CTLs in the melanoma metastasis of patient 1. Immunohistochemistry of paraffin sections from the melanoma tumor of patient 1, showing melanoma cells that stain positive for the proteins gp100 (A) and Melan-A (B). Original magnification 200x. Small inserts show tetramer-analysis. $CD8^+$ T cells (horizontal axis) are plotted against $gp100^{154\text{-}162}$-epitope and Melan-$A^{26\text{-}35}$-epitope tetramers (vertical axis) showing 0.32% gp100 (A) and 1.7% Melan-A (B) specific CTLs (see also table 1).

Concluding remarks

Melanoma-associated vitiligo is more often seen in patients who undergo immunotherapy. We demonstrate that immunotherapy against gp100 and tyrosinase antigens can induce specific and functional CTLs that invade both melanoma and vitiligo lesions. This directly links the occurrence of vitiligo to the immuno-therapeutic intervention.

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Chapter 7

First clinical experience: osteosarcoma patient vaccinated with monocyte-derived dendritic cells, pulsed with tumor specific MAGE-peptides

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Abstract

Vaccinations of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells (DCs) are applied in the clinic in experimental settings to induce a tumor-specific immune response. A concern relevant to the use of whole tumor cell-derived material is the risk to induce autoimmune disease. Specific immune monitoring is more challenging since the epitopes involved in the immune response are not known.

Here we report our experience of the first pediatric patient vaccinated with mature DCs loaded with tumor associated peptides. A 17-year-old patient with a metastasized osteosarcoma had progressive tumor growth during standard chemotherapy. Autologous mature DCs were generated from peripheral blood monocytes. The DCs were pulsed with the immunogenic protein keyhole limpet hemocyanin (KLH) and MAGE-A1 peptides. $15x10^6$ DCs were administered intradermally and $30x10^6$ intravenously every two weeks for a total of three vaccinations. An antibody response against KLH was detected in the serum of the patient after vaccination. No specific T cell responses against the MAGE-A1 were detected in peripheral blood or in the delayed type hypersensitivity skin-reaction. The patient had an ongoing progression of the osteosarcoma.

Our results demonstrate that it is feasible to generate fully matured, peptide loaded DCs in sufficient numbers. The vaccine induced an KLH specific immune response but did not generate a peptide specific T cell response or tumor regression.

Introduction

Osteosarcoma is a primary bone malignancy generally affecting adolescents with 60% of cases occurring before the age of 25 years and the peak incidence at 15 years. Aggressive chemotherapy dramatically improves prognosis for patients with non-metastatic osteosarcoma of the extremities. However, prognosis is still poor for patients with metastatic disease at diagnosis or those that do not respond to standard therapy.[1] New treatment strategies to improve survival rates for patients with a poor prognosis include radiotherapy, introduction of new chemotherapies and immune-based therapies.[2,3]

Dendritic cells (DCs) are professional antigen presenting cells that play a crucial role in both the initiation and modulation of immune responses. DCs have the unique ability to take up and process antigens in the peripheral blood and tissues. In the presence of inflammation or endogenous danger-signals, the immature DCs undergo a maturation process. Mature DCs subsequently migrate to draining lymph nodes, where they present antigen to resting lymphocytes.[4] Our increased understanding of DC biology and the possibility to obtain large numbers of DCs *in vitro* from isolated monocytes has boosted the use of DCs in tumor immunotherapy.[5,6]

Most of the DC-based vaccines have been completed in adults. To date, three groups report promising clinical experiences using DCs to treat pediatric patients with solid tumors.[7-9] In all three studies patients are treated with DCs that are pulsed with either whole tumor lysate or whole tumor RNA. The main advantages of using whole tumor is that 1) multiple tumor epitopes can be processed and presented by the DC which reduces the possibility of immune escape, 2) the tumor-epitopes do not necessarily have to be characterized and 3) it expands the clinical application as there is no MHC-restriction for patient inclusion.[10] A concern relevant to the use of whole tumor cell-derived material is that the generated immune response could be less specific and may cause autoimmune disease. Specific immune monitoring is also more difficult since the epitopes involved are not known.[11]

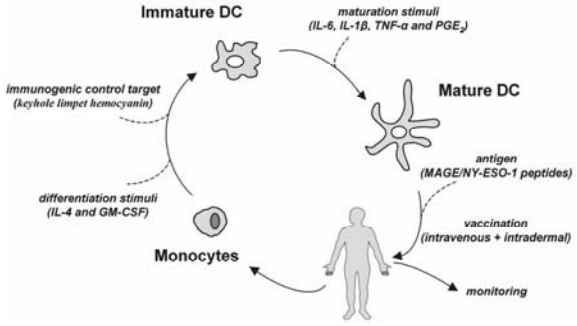
We recently reported that osteosarcomas highly express tumor specific MAGE and NY-ESO-1 antigens.[12] Several immunogenic peptides encoded by the MAGE and NY-ESO-1 genes presented by HLA molecules at the surface of tumor cells have been identified.[13] Tumor specific peptides have been extensively used for loading of DCs to induce an immune response against those targets. The main advantage of using a defined peptide is to generate an immune response that is specific for that epitope. This approach minimizes the risk of autoimmunity and facilitates immuno-monitoring as the immune target is defined. However, because of the MHC restriction of peptides, they are only applicable to MHC-matched patients. In a clinical feasibility trial we investigate peptide-specific DC vaccinations in pediatric patients with solid tumors.

Results

Inclusion

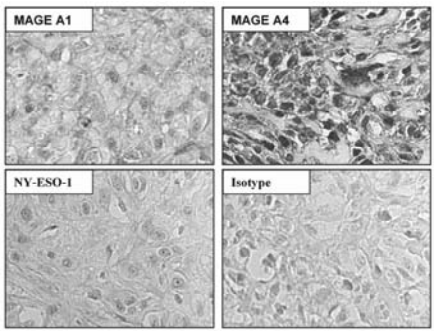
A 17-year-old male diagnosed with a high grade osteosarcoma of the distal femur had progressive tumor growth during treatment with cisplatin, anthracycline and methotrexate. Since the patient also not responded to ifosfamide treatment he failed all standard treatment. After approval of our Institutional Review Board and informed consent of both patient and parents we included the patient for the peptide-specific DC vaccination protocol (figure 1). From freshly frozen tumor biopsies RNA was isolated for expression analysis of MAGE and NY-ESO-1 antigens.[12,14] The primary osteosarcoma expressed MAGE-A1, MAGE-A4 and NY-ESO-1 RNA which was confirmed on protein level using immunohistochemistry (figure 2). Several immunogenic peptides encoded by the MAGE-A1, MAGE-A4 and NY-ESO-1 genes have been identified and are clinically grade available.[13] Linking the peptide-database to the HLA-expression of our patient (HLA-A03xA03; B07xB39; DR01xDR15), we could only select 1 peptide for DCs pulsing: the MAGE-A1 peptide *SLFRAVITK* presented in HLA-A03.[15]

Figure 1



DC-vaccination protocol.
Monocytes isolated from peripheral blood are differentiated with GM-CSF and IL-4 into immature DCs, loaded with KLH and further matured using pro-inflammatory cytokines. Mature DCs are pulsed with tumor specific peptides derived from cancer germline genes and administered intravenously and intradermally.

Figure 2



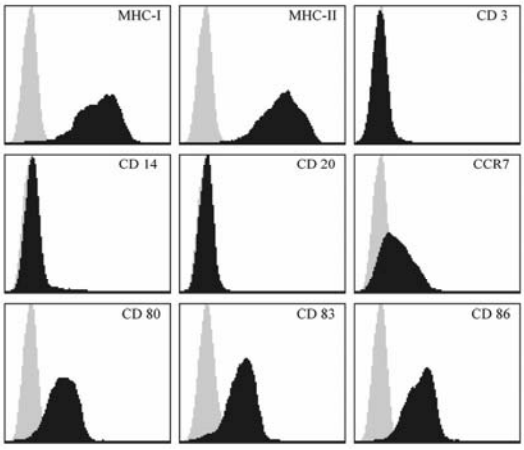
Cancer germline gene expression in primary osteosarcoma. Immunohistochemistry was performed on paraffin embedded tissue sections. Strong immunoreactivity was seen with monoclonal antibodies MA454 (anti-MAGE-A1) and 57B (anti-MAGE-A4). Less reactivity was seen with the E978 clone (anti-NY-ESO-1).

Leukapheresis and DC vaccine preparation

From a 9 liter apheresis, $7.7x10^9$ peripheral blood mononuclear cells were harvested. Monocytes were isolated with plastic adherence. For generation of clinical-grade DCs, monocytes were cultured with GM-CSF (800 U/ml) and IL-4 (500 U/ml), according to our standard operating procedures in an GMP-facility.[16] DCs were loaded with the foreign protein KLH, for immunomonitoring purposes. We harvested $920x10^6$ monocyte derived immature DCs that were further matured at day 6 with recombinant TNF-α (10 ng/ml), prostaglandin E2 (10 µg/ml) IL-1β (5 ng/ml) and IL-6 (15 ng/ml) as described previously.[17,18] Mature DCs were pulsed with 1mM peptide *SLFRAVITK* for 3 hours.

Before vaccination, the phenotype of the DCs at day 8 was analyzed using flow cytometry. The DCs highly expressed MHC class I and MHC class II. They further expressed the activation marker CD83, the costimulatory molecules CD80 and CD86, and the chemokine receptor CCR-7 (figure 3), which represents a fully mature phenotype of the DCs. The DC-vaccine was not contaminated with T cells (CD3 negative), B cells (CD20 negative) or monocytes (CD14 negative). DCs were injected every two weeks for a total of three vaccinations. Each vaccination the patient received $15x10^6$ and $30x10^6$ peptide-loaded mature DCs intradermally and intravenously, respectively.

Figure 3

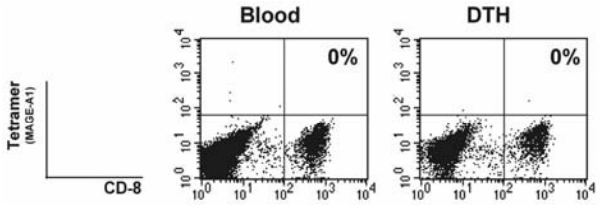


Dendritic cell phenotype. MHC class I, MHC class II, CD3, CD14, CD20, CCR7, CD80, CD83 and CD86 expression on the matured monocyte-derived DCs used for vaccination as measured with flow-cytometry (gray histograms represent the isotype-matched controls).

Immunomonitoring

After two DC-vaccinations the patient generated an antibody response against the control protein KLH. The KLH-specific antibody-titer further increased after completion of the third vaccination (data not shown). For the delayed type hypersensitivity assay (DTH), $1x10^6$ DCs loaded with the MAGE-A1 peptide were administered intradermally 2 weeks after the third vaccination. Maximal induration, 13mm, was measured two days later. T cell culture from DTH biopsies was performed in low dose IL-2 (100 U/ml), for 2 weeks without *ex vivo* restimulation with antigen as described previously.[19] In both peripheral blood and in the DTH no MAGE-A1 specific T cells were detected, as measured with tetramer-analysis (figure 4). During DC-vaccinations the patient continued to have progressive disease, he died 6 months later due to lungmetastases.

Figure 4



Tetramer-analysis. None of the $CD8^+$ T cells *(x-axis)* derived from peripheral blood or the delayed type hypersensitivity-reaction are tetramer positive for the MAGE-A1 peptide *(y-axis)*.

Conclusion

In this study we demonstrate that it is feasible to produce mature monocyte-derived DCs pulsed with peptides for vaccination in a pediatric patient with high risk osteosarcoma. No toxicity was observed during treatment. Although vaccine-specific immunity was induced, no peptide specific T cells were detected and disease progression could not be halted.

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Chapter 8

Toll-like receptor signaling on Tregs:

to suppress or not suppress?

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Summary

To balance self-tolerance and immunity against pathogens or tumors the immune system depends on both activation mechanisms and down regulatory mechanisms. Immunologists have long been focusing on the activation mechanisms and a major breakthrough was the identification of the Toll-like receptor (TLRs) family of proteins. TLRs recognize conserved molecular patterns present on pathogens, including bacteria, viruses, fungi and protozoa. Pathogen recognition via TLRs activates the innate as well as the adaptive immune response. The discovery of a suppressive T cell subset, that constitutively expresses the IL-2 receptor α-chain (CD25), has boosted studies investigating negative regulation of immune responses. It is now well appreciated that these regulatory T cells (Tregs), play a pivotal role in controlling immune function. Interestingly, recent studies revealed that TLR2 signaling affects Treg expansion and function. This review will focus on the presence and influence of different TLRs on T-lymphocytes, including Tregs, and their role in cancer.

Introduction

Induction of protective T cell responses requires naive T cells to receive signals via their T-cell receptor (TCR), co-stimulatory molecules and cytokine receptors. These signals can be provided by professional antigen presenting cells, like activated dendritic cells (DCs). DCs are stimulated when they encounter pathogen associated molecular patterns (PAMPs) that are recognized by pathogen recognition receptors, such as Toll-like receptors (TLRs). TLR engagement alerts the immune system for danger and leads to the activation of innate immune cells,[1, 2] e.g. production of pro-inflammatory cytokines, induction of phagocytosis and other innate effector mechanisms. Furthermore, TLR triggering induces DC maturation which is essential for the induction of adaptive immune responses.[1] DC maturation leads to the upregulation of co-stimulatory molecules and MHC molecules, secretion of immune modulatory cytokines (i.e. IL-12, IL-23) and chemokines, and enhanced migration from the periphery to draining lymph nodes.[2-4] Within mice and humans thirteen TLRs have now been identified that recognize distinct conserved PAMPs.[3, 5] For example, TLR4 and TLR5 recognize the gram-negative bacterial component LPS and the bacterial flagellin protein respectively.[4-6] TLR2 can interact with either TLR1 or TLR6.[4, 5] The heterodimer TLR1/2 recognizes bacterial triacyl lipopeptides, while TLR2/6 recognizes bacterial diacyl lipopeptides. Recently, profilin on uropathogenic *E.coli* and *T.gondii* was identified as the ligand for TLR11.[7, 8] These bacteria sensing TLRs are largely located on the cell surface of immune cells. In contrast, TLR3, TLR7, TLR8 and TLR9 are present inside immune cells and can recognize nucleic acids like RNA and DNA or derivates thereof.[4-6] They sense the presence of intracellular pathogens or virally infected cells following phagocytosis. The ligands for TLR10, TLR12 and TLR13 are as yet unknown.

Besides pathogen derived exogenous ligands, some TLRs can also become activated by recognition of so-called endogenous ligands. TLR2 and TLR4 have both been reported to interact with heat shock proteins[9-11] and necrotic cells, TLR3 with mRNA.[12] TLR4 has also been proposed to interact with fibronectin, fibrinogen, and murine β-defensin 2.[6, 13, 14] Finally, TLR9 has been shown to become activated by chromatin-IgG complexes found in the autoimmune disease SLE.[15] The finding that TLRs can recognize endogenous ligands, especially ligands that are released following tissue destruction/pathology, as well as PAMPs indicates they are not only key molecules in immunity against micro-organisms but also may play a role in autoimmune diseases and cancer.[6]

Immune suppression: regulatory T cell subsets

To prevent extensive immune-mediated tissue damage or auto-immune diseases, the initiation, expansion and retraction of effector T cell (Teff) responses need to be closely controlled. Here for, multiple feedback control mechanisms are in place within the Teff itself, like induction of suppressor of cytokine signaling (SOCS) genes and activation induced cell death following T cell activation.[16, 17] In addition, Teff immune responses are highly regulated by immune suppressive regulatory T cell subsets. The significance of regulatory T cells (Tregs) in maintaining immune homeostasis is illustrated by the development of autoimmune symptoms in individuals lacking functional Tregs.[18] Furthermore, the occurrence of autoimmunity in spontaneous autoimmune models in mice could be prevented upon transfer of Tregs,[19, 20] while temporal depletion of Tregs improved cancer vaccine efficiency by enhancing Teff responses.[21]

Several distinct immunosuppressive Treg subsets have been described, which can be broadly subdivided into two groups, 1) cells that originate from the thymus,[22] referred to as 'naturally occurring Tregs', and 2) Tregs that have been induced in the periphery, also called 'adaptive Tregs'. The best characterized Tregs are the naturally occurring $CD4^+CD25^+$ Tregs that constitute 5 to 15% of the total $CD4^+$ T cell population.[22] Once activated, the $CD4^+CD25^+$ Tregs are able to suppress T cell proliferation and cytokine production as well as antigen presenting cell function.[23] The suppressive activity of these cells requires TCR triggering by MHC class II molecules presenting either self or non-self peptide epitopes. $CD4^+CD25^+$ Treg mediated suppression is known to be antigen non-specific and involves cell-contact dependent mechanisms.[24] Naturally occurring Tregs express the transcription factor forkhead box protein (FoxP3),[25, 26] which has been shown to be induced by the cytokine TGF-β.[27, 28] Recently, the AKT signaling pathway has been identified as a strong repressor of Treg differentiation in the thymus by diminishing TGF-β-induced FoxP3 expression. AKT mediated signals thus represent a major determinant with broad impact on the onset of Treg specification.[29] Recently Ono *et al.* found that FoxP3 physically interacts with the transcription factor AML1/Runx1, thereby preventing IL-2 and IFNγ production by Tregs while inducing Treg-cell-associated molecules and suppressive activity.[30] The importance of FoxP3 in Treg development and function was further demonstrated in FoxP3 knockout mice as well as scurfy mice that carry a natural mutation in the FoxP3 gene. Interestingly, both mice show autoimmune symptoms resembling the human immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome that is caused by a mutation in the human FoxP3 gene.[18] Furthermore, transduction of FoxP3 into naïve $CD4^+$ T cells resulted in a suppressive phenotype in mice.[26, 31] Although several other markers have been identified in human and/or murine

$CD4^+CD25^+$ Tregs, like cytotoxic T-lymphocyte activation antigen (CTLA-4),[32] Glucocorticoid-induced TNFR family-related gene (GITR),[33] integrin $\alpha_E\beta_7$ (CD103),[34] CCR8 and the absence of CD127,[35] FoxP3 represents the most specific marker for naturally occurring Tregs available to date.

$CD4^+$ regulatory T cells that develop in the periphery are referred to as adaptive Tregs. At least two types of adaptive Tregs have been characterized. Type 1 regulatory T cells (Tr1) arise after repeated TCR stimulation in the presence of IL-10.[36, 37] More recently, IL-27 produced DC upon interaction with FoxP3 expressing Tregs was shown to be a key factor in the generation of Tr1 cells which could be further enhanced by TGF-β.[38] Tr1 cells have been identified in humans and in mice, and are able to inhibit T cell responses *in vitro* and *in vivo.*[36] Tr1 cells do not express FoxP3 and mediate suppression by secreting high amounts of IL-10.[36] Altered Tr1 function has been reported for patients with multiple sclerosis,[39] and adoptive transfer of Tr1 cells inhibited the development of murine experimental allergic encephalomyelitis in vivo.[40] Th3 cells represent the second subset of adaptive Tregs. They differentiate from naive CD4 T cell precursors by repeated TCR stimulation in combination with high amounts of TGF-β.[41, 42] Th3 cells also secrete high amounts of TGF-β themselves, thereby suppressing immune responses.[41, 42] Ag-specific TGF-β producing Th3 cells have recently been shown to be important in inducing and maintaining peripheral tolerance by driving the differentiation of adaptive Ag-specific $FoxP3^+$ regulatory cells in the periphery in mice.[43, 44] We note that also human $CD4^+CD25^+$ Tregs expressing $\alpha_4\beta_7$ integrin or $\alpha_4\beta_1{}^+$ have been shown to induce Tr1-like and Th3-like T suppressor cells from naïve $CD4^+$ T cells respectively.[45]

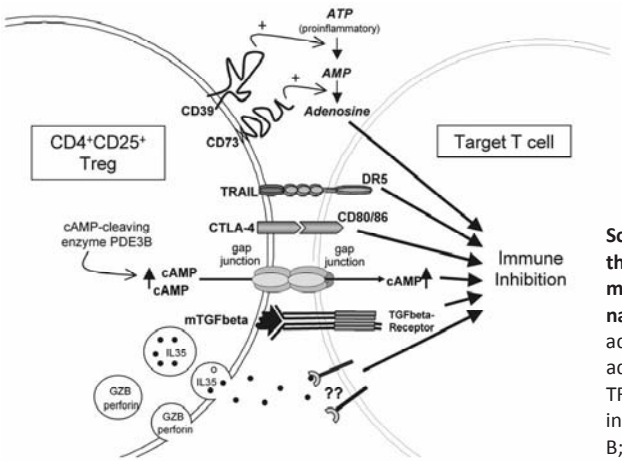
Aside the $CD4^+$ regulatory T cell subsets expressing the αβ TCR, recent studies showed that $CD8^+CD122^+$ regulatory T cells,[46] and T cells carrying the γδ TCR can also have immune suppressive functions. T cells expressing the γ and δ TCR chains are divided into two different subsets, namely Vδ1 (γδ1 T cell or intraepithelial lymphocytes) and Vδ2 (residing in peripheral blood).[47] γδ1 T cells isolated from breast tumor tissue were shown to be highly suppressive in a trans-well system, but the soluble factor causing the suppression remains to be identified.[47] These tumor-infiltrating γδ1 T cells do not express CD25, GITR, or FoxP3.

In conclusion, the field of regulatory T cells is still expanding, and new regulatory subsets are likely to be discovered in the coming years. The existence of multiple Treg subsets underscores the importance of immune suppressive cells within the immune system, and addressing their role in tolerance and immunity will be an intense area of future research.

Suppressive mechanisms of naturally occurring Tregs

Tr1, Th3 and γδT cells are known to suppress via secretion of soluble factors, like anti-inflammatory cytokines. Naturally occurring $CD4^+CD25^+$ Tregs suppress via contact-dependent mechanisms. In the past few years, multiple cell contact-dependent mechanisms exploited by these Tregs have been reported (figure 1). Cell contact-dependent suppression was shown to involve CTLA-4 and membrane-bound TGF-β (mTGF-β) expressed on the cell surface of $CD4^+CD25^+$ Tregs,[35, 48] *in vitro* secretion of granzyme B[49] or perforin,[50] and through modulation of IL-2 responsiveness.[35, 51] In line with the finding that neither of the aforementioned suppressive mechanisms could entirely explain Treg suppression, several new suppression mechanisms have recently been defined. In mice, $CD4^+CD25^+FoxP3^+$ Tregs preferentially express the ectonucleotidases CD39 and CD73 on their cell-surface and their expression is amplified and stabilized by FoxP3.[52, 53] CD39 degrades nucleoside tri- and diphosphates like ATP into adenosine monophosphates (AMP) and CD73 catabolizes the conversion of AMP into adenosine. The presence of extracellular ATP is regarded as an indicator for tissue damage and can function as a natural immune adjuvant and danger signal by binding to the purinergic receptors. In contrast, adenosine is known to exert an immunosuppressive effect on immune cells, like inhibition of proliferation and TNF or IFNγ synthesis by Th1 cells,[52-54] upon binding to adenosine receptors. Thus, CD39 mediated removal of the pro-inflammatory ATP and its conversion into immunosuppressive AMP by CD73 represents another mechanism by which $CD4^+CD25^+FoxP3^+CD39^+CD73^+$ Tregs can suppress immune responses.[52, 53]

Figure 1



Schematic representation of the different suppressive mechanisms employed by naturally occurring Tregs. ATP, adenosine triphoshpate; AMP, adenosine monophosphate; TRAIL, TNF-related apoptosis inducing ligand; GZB, granzyme B; IL-35, Interleukin-35.

In vitro and *in vivo* activation of the TRAIL/DR5 pathway is a known mechanism to induce apoptosis. Recently, TRAIL, or TNF-related apoptosis inducing ligand, was found to be upregulated upon Treg activation, while activated Teffs express increasing levels of DR5.[55] Addition of DR5-blocking antibodies significantly reduced the suppressive capacity of the Tregs in vitro and in vivo.[55]

Proteomic analysis of human $CD4^+CD25^+$ regulatory T cells revealed galectin-10 to be a specific intracellular marker of $CD4^+CD25^+$ Tregs.[56] Further analysis revealed that siRNA mediated downregulation of galectin-10 abrogated Treg suppressive capacity, but further characterization is necessary to elucidate the exact physiological role in suppression.

Another mechanism became apparent following the discovery that the cAMP-cleaving enzyme phosphodiesterase 3B (PDE3B) is strongly reduced in Tregs as compared to conventional $CD4^+$ T cells. As a consequence Tregs contain elevated levels of cAMP, a second messenger known to regulate a wide variety of cellular functions in a large group of cell types.[57] In T lymphocytes increased levels of endogenous cAMP inhibit cell activation, cytokine production, and cell proliferation by interfering with the activation of Ras and Rap1.[57] Bopp *et al*. now demonstrated that contact-dependent suppression by naturally occurring Tregs can occur via a well-known mechanism, namely the intercellular transport of cAMP via gap junctions.[58] Upon Treg interaction with a target cell, cAMP levels within the target cell increased resulting in immune suppression, which could be blocked by addition of a gap junction inhibitor.[58] These two examples link the previously described role of nucleotide catabolites in immune regulation to the cell-contact dependent suppressive activity of Tregs.

Functional genomics analysis comparing $CD4^+CD25^+FoxP3^+$ Tregs with $CD4^+CD25^-$ Teff also led to the discovery of the inhibitory cytokine IL-35, a novel member of the IL-12 heterodimeric cytokine family.[59] Epstein-Barr-virus-induced gene 3 (Ebi3), was shown to be preferentially expressed in the Treg subset along with IL-12α chain (or p35) to form the heterodimer IL-35. Subsequent in vitro experiments showed that Tregs from Ebi3 knockout or IL-12α knockout have a significantly reduced suppressive capacity.[59] Teffs retrovirally transduced with IL-35 gained suppressive activity and also recombinant IL-35 inhibited Teff proliferation. Subpopulations of γδ T cells and $CD8^+$ T cells may also express small amounts of IL-35, suggesting that IL-35 may be involved in their regulatory potential as well. Whether or not IL-35 can be induced in $FoxP3^+$ Th3 cells is not known. The discovery of IL-35 secretion as an additional factor required for maximal Treg mediated immune suppression is intriguing, but also raises questions regarding contact dependency of Treg mediated suppression and the target cells expressing the putative IL-35 receptor.

The presence of multiple different suppressive mechanisms exploited by Tregs further raises the question which suppressive mechanism(s) are most important for inhibition of a particular function in a given target cell or pathological condition.

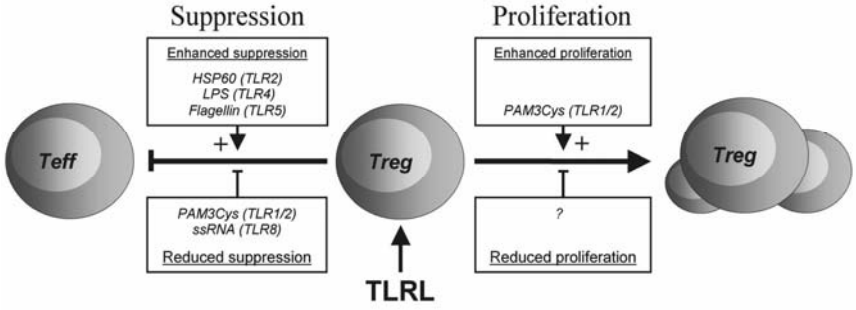
Modulation of Treg function

Tregs play a central role in the suppression of immune reactions and prevention of autoimmune responses harmful to the host. However, during acute infection, Tregs might hinder effector T cell activity directed towards the elimination of the pathogenic challenge. Therefore, Treg mediated suppression needs to be tightly controlled. Control of Treg function is known to occur through cytokines like IL-1, IL-6 and IL-12, and multiple co-stimulatory molecules expressed by antigen presenting cells.[60, 61] These cytokines and co-stimulatory molecules are efficiently induced upon TLR stimulation of APC and act either by direct stimulation of Treg proliferation and/or inhibition of Treg suppression or indirectly by rescuing Teffs from Treg mediated suppression. Another key cytokine that supports Treg development and maintenance is IL-2. Furthermore, IL-2 plays a dominant role in regulating Treg mediated suppression.[61, 62] IL-15, which signals through the common IL-2 receptor β and γ chain, is able to substitute IL-2 as a growth factor in vitro, while IL-4 and IL-7 can act as growth and survival factors, respectively.[60]

More recent TLR expression profiling studies revealed that multiple TLRs are expressed in $CD4^+$ T cells as well as $CD4^+CD25^+$ Tregs.[6, 60, 63-65] Interestingly, murine and human $CD4^+CD25^+$ Tregs express higher levels of TLR4, TLR5, TLR7, and TLR8 in comparison with $CD4^+$ Teffs.[6, 60, 63, 64]. Several independent studies have now highlighted the importance of TLRs on $CD4^+CD25^+$ Tregs (figure 2). TLR5 is expressed on both $CD4^+$ Teffs and $CD4^+CD25^+$ Tregs.[66] Interaction of flagellin with its receptor TLR5 on Teffs increased their proliferation and production of IL-2 while on Tregs flagellin/TLR5 increased their suppressive capacity.[63, 66] The influence of TLR4 on Tregs is not yet clear. Initially, it was reported that LPS could enhance murine Treg mediated suppression by binding to TLR4 on Tregs,[64] but this direct effect of LPS on purified Tregs could not be confirmed by others.[65-67] Nevertheless, the confined expression of TLR4 on Tregs, warrants further examination of the effects of TLR4 ligands, including endogenous TLR4 ligands, on Tregs. We reported an important role for TLR2 in regulating murine Treg mediated suppression.[67] TLR2 activation on Tregs using the synthetic ligand PAM3Cys, in combination with IL-2 and TCR triggering, can induce Treg proliferation and results in temporal loss of suppression. Upon removal of the TLR2 ligand, the Tregs regained their suppressive function.[67] Applying TLR2 knockout and MyD88 knockout mice showed that these effects on Treg function were indeed TLR2 and MyD88 dependent. Similar findings were reported by Liu *et al.* [68]. They further

suggested downregulation of FoxP3 as a putative mechanism for the abrogation of Treg suppression. Surprisingly, using the endogenous TLR2 ligand hsp60, opposite effects of TLR2 triggering on Tregs were observed by Zanini-Zhorov *et al.* Hsp60 activated Tregs enhanced their suppressive capacity by both cell-contact dependent mechanisms and TGF-β and IL-10 production.[9] This discrepancy could possibly be explained by the nature of the TLR-ligands used, PAM3Cys being a TLR1/2-ligand and hsp60 a TLR2/? ligand, the concentrations of the ligands used or differences in the way endogenous and exogenous ligands interact with TLR2. TLR8 is strongly and preferentially expressed on human Tregs as compared to human Teffs.[69] Triggering of TLR8 on Tregs resulted in the specific abrogation of suppression without affecting Treg proliferation, while no effects on human Teffs were observed. Applying short interfering RNA technology to knock-down TLR8 in Tregs completely blocked the effect demonstrating a crucial role for TLR8.[69]

Figure 2



Effects of TLR-ligands proposed to directly modulate the function of naturally occurring Tregs upon direct interaction of the TLR-ligand with the Treg. Pre-treatment of Tregs with either endogenous HSP60 (TLR2), LPS (TLR4) or Flagellin (TLR5) has been reported to enhance the Treg suppressive capacity. In contrast, PAM3Cys (TLR1/2) and ssRNA (TLR8) abrogate the suppressive capacity. The exposure of Tregs to PAM3Cys induces Treg proliferation and is mediated via TLR1/2. The effect of signaling via TLR6,-7 or the effect of combinations of TLR-ligands on Tregs is not known. CpG, guanosine-containing DNA oligonucleotides; HSP60, endogenous 60kDa heat shock protein; LPS, lipopolysaccharide; ssRNA, single stranded RNA.

The positive and negative effects of TLR-ligands on Tregs themselves are intriguing, but further research is required to fully decipher the role of TLR triggering on Tregs. Crucial questions regarding the dynamics of TLR expression on immune suppressive Treg subsets upon inflammation or in relation to the type of pathogen encountered are largely unexplored. Aside pathogen-derived TLR-ligands, it will also be important to elucidate the impact of endogenous TLR-ligands on Tregs to shed light on the multifactorial regulation of Treg homeostasis in health and disease.

Tregs, TLRs and cancer

Multiple studies have shown that immune suppressive T cells can infiltrate tumors and dampen anti-tumor immune response in mice.[70, 71] Increased levels of Tregs have been documented in the peripheral blood of cancer patients and especially in the local tumor microenvironment.[72] Naturally occurring $CD4^+CD25^+$ Tregs as well as adaptive $CD25^+FoxP3^+$ Tregs, Tr1- and Th3 cells, have all been detected in tumors.[51] Next to these $CD4^+$ suppressor T cells, other suppressive cell types reported to be involved in tumor-immune escape are IL-10 secreting $CD8^+$ cells,[73] invariant NKT cells[74, 75] and γδ T cells.[47] Wang *et al.* succeeded to isolate human tumor infiltrating Tregs and identified LAGE-1 and ARTC1 as the first natural tumor ligands for these Tregs.[76, 77] Collectively, these findings could possibly explain why even in tumors found to be infiltrated with leukocytes,[78-80] tumor progression is seemingly unhindered.

The detrimental effect of Tregs in anti-tumor immunity is emphasized by murine cancer models showing that Treg depletion with monoclonal antibodies against CD25 lead to significantly increased anti-cancer immunity.[81-85] Moreover, Treg depletion improves the efficacy of anti-cancer vaccines. As TLRs provide an important link between innate and adaptive immunity, TLR-ligands are increasingly applied in cancer vaccines.[86] However, besides innate immune cells and now T lymphocytes, also non-immune cells like epithelial cells and keratinocytes have been shown to express TLRs. Moreover, recent reports indicate that TLRs can also be expressed on tumor cells.[87-90] It might be especially rewarding to investigate the effects of TLR-ligands on the function of different inhibitory T cell subsets. So far, application of TLR8-ligands have been shown to enhance immune function of DC and at the same time reduce the suppressive capacity of human Tregs.[69] In contrast, the immune stimulatory potential of LPS and flagellin, might be counteracted by the direct enhancement of the suppressive function of murine Tregs via TLR4 and TLR5, respectively.[63, 64, 66] TLR2 plays a crucial role in both Treg expansion[67] and the suppressive capacity of Tregs.[68] Hence, stimulation via this receptor may lead to temporal immune stimulation but also a profound increase in the number of Tregs. Selecting the optimal combination of TLR-ligands for a given vaccine may turn out to be a crucial component in maximizing the anti-tumor immune response.

In summary, understanding the functional control of immune suppressive T cells, including the role of TLR signaling, may offer new opportunities to shift the balance between immunity and tolerance. This, and the identification of specific targets on immune suppressive T cells that allow their elimination from the tumor microenvironment, represent some of the major challenges towards the development of effective cancer immunotherapy.

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Chapter 9

Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors

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Neuro-oncology: in press

Abstract

The brain is a specialized immune site representing a unique tumor microenvironment. The availability of fresh brain tumor material for ex-vivo analysis is often limited as large parts of many brain tumors are resected using ultrasonic aspiration. We now analyzed ultrasonic tumor aspirates as a bio-source to study immune suppressive mechanisms in 83 human brain tumors. Lymphocyte-infiltrates in brain tumor tissues and ultrasonic aspirates were comparable with respect to lymphocyte content and viability. Applying ultrasonic aspirates, we detected massive infiltration of $CD4^{+}FOXP3^{+}CD25^{high}CD127^{low}$ regulatory T cells (Tregs) in glioblastomas (n=29) and metastatic brain tumors (n=20). No Treg accumulation was observed in benign tumors such as meningiomas (n=10) and pituitary adenomas (n=5). A significant Treg increase in blood was only seen in patients with metastatic brain tumors. Tregs in high-grade tumors exhibited an activated phenotype as indicated by decreased proliferation and elevated CTLA-4 and FoxP3 expression relative to blood Tregs. Functional analysis showed that the tumor derived Tregs efficiently suppressed cytokine secretion and proliferation of autologous intratumoral lymphocytes. Most tumor infiltrating Tregs localized in close proximity to effector T cells, as visualized by immunohistochemistry. Furthermore, 61% of the malignant brain tumors expressed programmed death ligand-1 (PD-L1), while the inhibitory PD-1 receptor was expressed on $CD4^{+}$ effector cells present in 26% of tumors. In conclusion, using ultrasonic tumor aspirates as a bio-source we identify Tregs and the PD-L1/PD-1 pathway as immune suppressive mechanisms in malignant but not benign human brain tumors.

Introduction

Brain tumors account for two percent of all cancers and result in a disproportionately high share of cancer morbidity and mortality. The World Health Organization (WHO) published a grading system that is used, in combination with clinical findings, to predict a response to therapy and outcome.[1] The most frequent primary brain tumors are meningiomas (WHO-grade I) and glioblastomas (WHO-grade IV and associated with a poor prognosis).[2] Brain metastases are tumors which originate elsewhere in the body and metastasize to the brain. Brain metastases account for more than 50% of all brain tumors in adults. The median survival of patients with brain metastases ranges from 2 to 8 months, despite maximal treatment.[3]

The idea that the brain is 'immunologically privileged' is being discarded, and replaced with appreciation that the brain is an immune specialized site under tight regulatory control.[4-6] Brain tumor infiltrating lymphocytes (TILs) provide evidence that the immune system is naturally involved in the immunosurveillance of brain tumors.[7,8] Tumors on the other hand create an immunosuppressive network by which they can escape from this immune attack.[9] Glioblastomas can escape immune regulation by secretion of immunosuppressive cytokines,[10] activation of negative regulatory pathways in lymphocytes,[11,12] evasion of immune recognition[13] and CCL2-dependent recruitment of CCR4 positive Tregs.[14]

The relative importance of Tregs in this immunosuppressive network is demonstrated in experimental mouse glioma models that show a dramatic influx of Tregs and that temporal Treg depletion markedly augmented anti-tumor immunity.[15-17] These data strengthen the view that Tregs create a tolerogenic environment that hampers anti tumor-immunity.

We examined the presence of $CD4^{+}FoxP3^{+}CD25^{high}CD127^{low}$ Tregs in patients with brain tumors. Large volumes of fresh brain tumor are needed to analyse the quantity, morphology and function of intratumoral Tregs. For the surgery of most brain tumors ultrasonic aspiration is a widely used technique next to normal resection.[18] The availability of fresh brain tumor for clinical research is limited as the resected tumor fragments are often small and needed for diagnostic purposes. The integration of a suction adapter into ultrasonic aspirators has made it possible to collect ultrasonic aspirated brain tumor material.[19] So far, tumor fragments removed by ultrasonic aspiration are used relatively infrequent in daily diagnostics[20] and, to our knowledge, not at all in clinical research.

In this study we show that the lymphocyte-fractions in ultrasonic aspirated material are similar to those in resected brain tumor tissue. Our data demonstrate that it is possible to isolate and study large numbers of viable Tregs from brain tumors that are surgically removed with ultrasonic aspiration. Using ultrasonic aspirated brain tumor as a new source for clinical research, we detected that Tregs accumulate specifically in high-grade brain tumors. The Tregs in the tumor microenvironment are fully activated and strongly suppress TIL-proliferation and cytokine production. We further show that a second important immune-regulatory pathway in brain tumors occurs via the PD-L1/PD-1 pathway. These immuno-modulating mechanisms may hamper spontaneous in-vivo immune responses and thereby contribute to the aggressive clinical behaviour of high-grade brain tumors.

Materials and Methods

Patients

We collected blood and freshly resected brain tumor from 83 brain tumor patients treated at the Radboud University Nijmegen Medical Centre or Canisius Wilhelmina Hospital (The Netherlands). All patients had histologically proven brain tumors, classified according to the WHO 2007 classification.[1] The primary brain tumors consisted of 21 WHO-grade I tumors (meningioma, n=10; pituitary adenoma, n=5; subependymal giant-cell astrocytoma, n=2; hemangioblastoma, n=1; craniopharyngioma, n=1; gangliocytoma, n=1 and schwannoma, n=1), 6 WHO-grade II tumors (atypical meningioma, n=3; hemangiopericytoma, n=2; oligodendroglioma, n=1), 6 WHO-grade III tumors (anaplastic oligodendrogliomas, n=4, anaplastic astrocytoma, n=1 and anaplastic ependymoma, n=1) and 31 WHO-grade IV tumors (glioblastoma, n=29; gliosarcoma, n=1). We further included 20 patients with a solitary metastatic brain tumor derived from lung (n=7), breast (n=3), kidney (n=2), colon (n=1), esophagus (n=1), melanoma (n=1), rectum (n=1), lymphoma (n=1) or unknown origin (n=3). Informed consent was obtained from all participants and the study was approved by our Institutional Review Board.

Preparation of brain tumor cell suspensions

Fresh brain tumor material was obtained by resection of the tumor tissue and/or ultrasonic aspiration with a Sonoca® 300 ultrasonic dissector/aspirator (Söring, Quickborn, Germany). Ultrasonic aspirates were collected in a closed system disposable suction bag (Serres, Kurikantie, Finland). Tumor fragments were filtered and washed to discard blood and suction fluid. Cell suspensions were made as previously described.[15] Briefly, mechanically disrupted brain tumor was incubated with collagenase type-IA (50 mg/ml), DNAse type-I (10 µg/ml) and trypsine inhibitor (1 µg/ml) in Hanks Balanced Salt Solution at 37°C and put on a Ficoll gradient.

Antibodies, flow cytometry and cell sorting

Flow cytometric analysis was performed with a FACS-Calibur (BD Biosciences, San Jose, CA) using directly labeled mAbs against CD4, CD8, CD25, CD127, CTLA-4, PD-1, PD-L1, PD-L2 (BD Pharmingen, San Diego, CA), FoxP3 (eBioscience, San Diego, CA) and Ki-67 (Dako, Glostrup, Denmark), all according to the manufacturers protocol. Tregs were defined as $CD4^{+}FoxP3^{+}CD25^{high}CD127^{low}$ cells (figure 1a) and percentage Tregs is defined as the number of $CD4^{+}FoxP3^{+}CD25^{high}CD127^{low}$ cells divided by the total of $CD4^{+}$ cells, times 100. To compare the level of FoxP3 and CTLA-4 expression between blood derived Tregs and Tregs in tumor tissue, mean fluorescence intensity of these markers was measured on both Treg subpopulations. Expression of Tregs in tumor tissue was calculated relative to the expression of blood derived Tregs which was set to 1. Following CD4 magnetic microbead selection (Miltenyi, Bergisch Gladbach, Germany), $CD4^{+}$ positive cells were sorted into $CD25^{+}CD127^{-}$ Tregs and $CD25^{-}CD127^{+}$ effector T cells using the Elite flow cytometric cell sorter.

Functional analysis of TILs in ultrasonic aspirated tumor
TILs isolated from ultrasonic aspirated material were cultured for 3 days with increasing concentrations of PHA. Supernatants were taken after 2 days and IFN-γ and IL-2 were measured by a cytometric bead array (Th1/Th2 Cytokine CBA 1; BD Pharmingen) according to manufacturers instructions. To measure proliferation, incorporation of ^{3}[H]thymidine was measured in duplicate in a ß-counter.

Suppression assay
The suppressive capacity of the Tregs was determined by addition of increasing numbers of freshly sorted brain tumor Tregs to $25x10^{3}$ brain tumor effector T cells (n=3). Cells were cultured for 4 days in the presence of $2.5x10^{3}$ CD3/CD28-beads (Invitrogen, Leek, The Netherlands). Cytokines in the supernatants were measured after 24 hours as mentioned above. Cell proliferation was monitored by ^{3}[H]Thymidine incorporation.

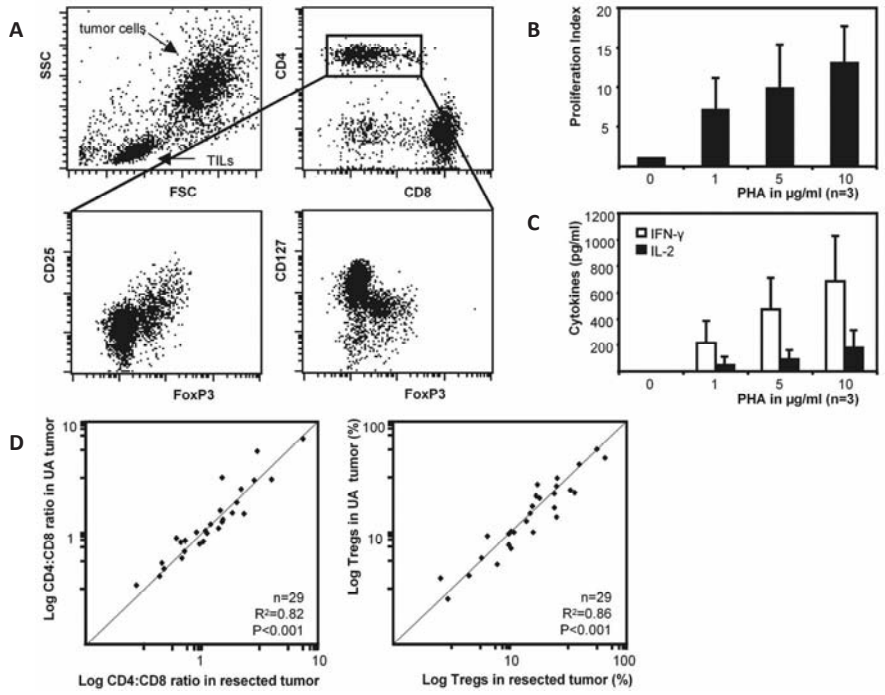
Immunohistochemistry
Immunochemistry was performed on 4 µm tissue sections of formalin-fixed paraffin-embedded tissue blocks. Sections were boiled for 20 min in citrate buffer (10 mM, pH 6.0). The following monoclonal antibodies (mAb) were used: 236A/E7 (eBioscience) against FoxP3 and SP7 (Labvision, Fremont, CA) against CD3 according to manufacturers protocol. Primary antibodies were detected with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) followed by an avidin–biotin complex system (ABC Elite, Vector Laboratories). Diaminobenzidine tetrachloride served as a chromogen.

Results

Ultrasonic aspirated brain tumor as a source to study tumor infiltrating lymphocytes

The effectiveness of the anti-tumor immune response is strongly influenced by the tumor microenvironment that can only be studied in fresh tumor material. We investigated whether ultrasonic aspirated brain tumor material can be used to study the tumor microenvironment. Hereto ultrasonic tumor aspirates were enzymatically digested and characterized by flow cytometry. Lymphocytes could be readily distinguished from tumor cells and were further subdivided into $CD8^+$ T cells, $CD4^+$ T cells and $CD4^+FoxP3^+CD25^{high}CD127^{low}$ Tregs (figure 1a). Isolated lymphocytes were still functional since they proliferated and secreted IFN-γ and IL-2 in response to increasing concentrations of phytohemagglutinin (PHA) (figure 1b,c).

Figure 1



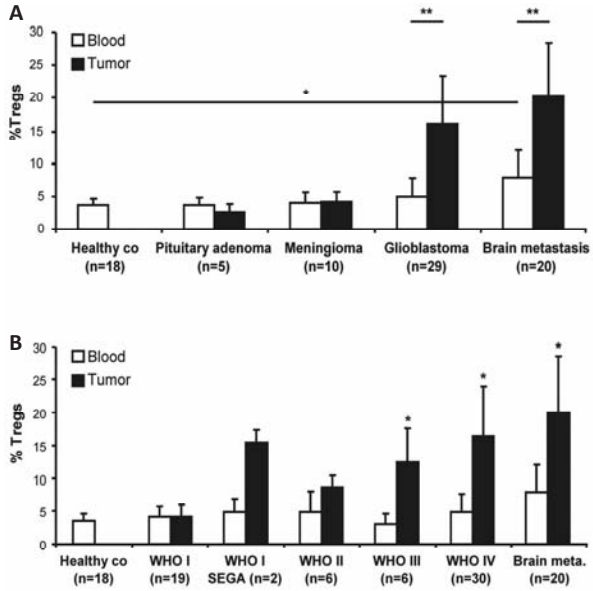
Analysis of tumor infiltrating lymphocytes. (a) Flow cytometry of ultrasonic aspirated glioblastoma material. Lymphocytes can be differentiated into $CD4^+$ cells (25%), $CD8^+$ cells (65%) and a population mainly consisting of B-cells (data not shown). $CD4^+$ cells can further be characterized into $CD4^+FoxP3^+CD25^{high}CD127^{low}$-cells and $CD4^+FoxP3^-CD25^{low}CD127^{high}$-cells. Glioblastoma TILs proliferate (b) and produce IFN-γ and IL-2 (c) upon increasing PHA-stimulation (the average of 3 glioblastoma patients is shown). (d) CD4:CD8-ratio (left scatterplot) and percentage Tregs (right scatterplot) in ultrasonic aspirated brain tumor and resected brain tumor are strongly correlated (n=29 tumors). UA=ultrasonic aspirate; PHA=phytohemagglutinin.

In 29 patients we were able to collect both tumor aspirates and resected brain tumor tissue allowing a direct comparison of lymphocyte-content in both tumor-sources. The $CD4^+$:$CD8^+$ T cell-ratio and the percentage of tumor infiltrating Tregs found in aspirated brain tumor fully correlated with the resected tumor tissue (figure 1d). These data indicate that ultrasonic aspirated tumor material provides an efficient and readily available source to study the brain tumor microenvironment.

Tregs specifically accumulate in high-grade brain tumors

Having shown that TILs from ultrasonic aspirated brain tumor faithfully represent resected tumor tissue, we compared Tregs in both tumor tissues and peripheral blood in 83 patients. The group is subdivided into patients with a primary brain tumor and patients with a solitary metastatic brain tumor derived from different primary tumors (hereafter called brain metastasis). The percentage of Tregs in the blood of patients with a primary brain tumor was not significantly increased compared to healthy volunteers. The amount of Tregs in brain tumors is strongly associated with the tumor-type (figure 2a). Low percentages of Tregs were detected in benign tumors such as pituitary adenomas (average 3.0%) and meningiomas (average 4.4%). Significantly higher percentages of Tregs accumulate in glioblastomas (average 16.5%, $P<0.003$). Moreover, Treg accumulation in brain tumors is positively correlated with the WHO malignancy-grade of the tumor (Figure 2b, $P<0.01$). The only exceptions are WHO-grade I subependymal giant-cell astrocytomas that also show an increase in the percentages of Tregs relative to blood.

Figure 2

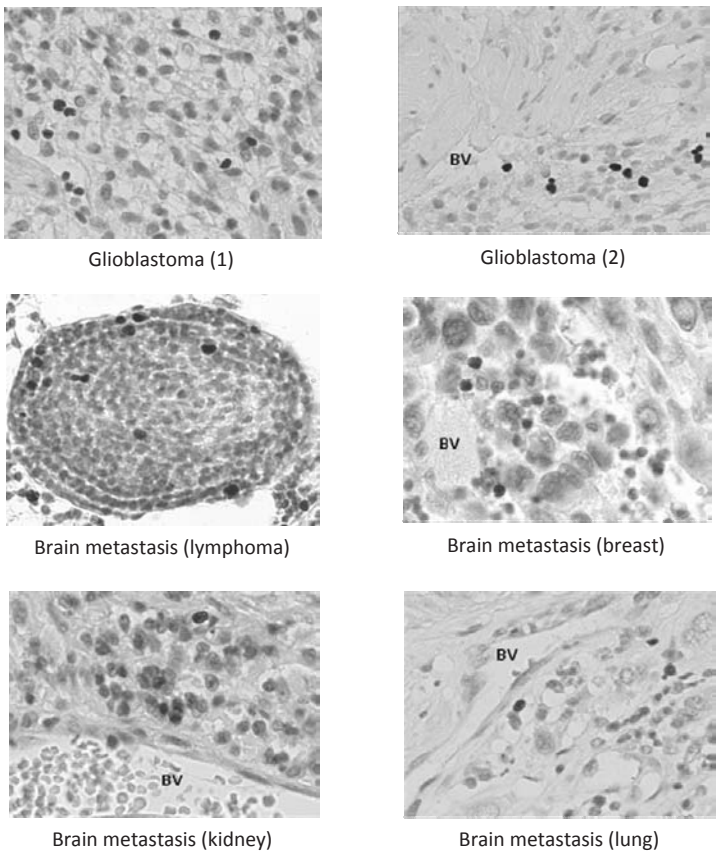


Tregs accumulate in high-grade brain tumors. (a) Percentage Tregs in blood (white bars) and tumor (black bars) of patients with brain tumors compared to healthy controls (*$p<0.01$, Student's t-test, **$p<0.003$, paired Student's t-test). (b) Percentage Tregs in brain tumors positively correlates with the WHO malignancy-grade (*$p<0.01$, Student's t-test). The WHO-grade I subependymal giant-cell astrocytomas are represented as a separate group to illustrate the relatively high percentages of Tregs in this tumor.

The highest level of intratumoral Treg recruitment was seen in patients with brain metastases, irrespective of the type of primary tumor, which suggests that Treg recruitment of metastatic brain tumors is a general phenomenon (figure 2). Interestingly, the percentage of Tregs in peripheral blood was also significantly increased in these patients with systemic disease. While on average 3.5% Tregs were detected in the blood of healthy controls, the blood of patients with brain metastases contained on average 7.9% Tregs (p=0.003).

To visualize Tregs in situ, we performed immuhistochemistry on paraffin embedded tumor tissue. The nuclear staining of FoxP3 in the Tregs could clearly be distinguished from the tumor cells and other TILs. Intratumoral Tregs were mainly localized perivascularly in close proximity to other lymphocytes. A smaller percentage of the Tregs infiltrated deep into the tumor tissue (figure 3).

Figure 3



Glioblastoma (1) Glioblastoma (2)

Brain metastasis (lymphoma) Brain metastasis (breast)

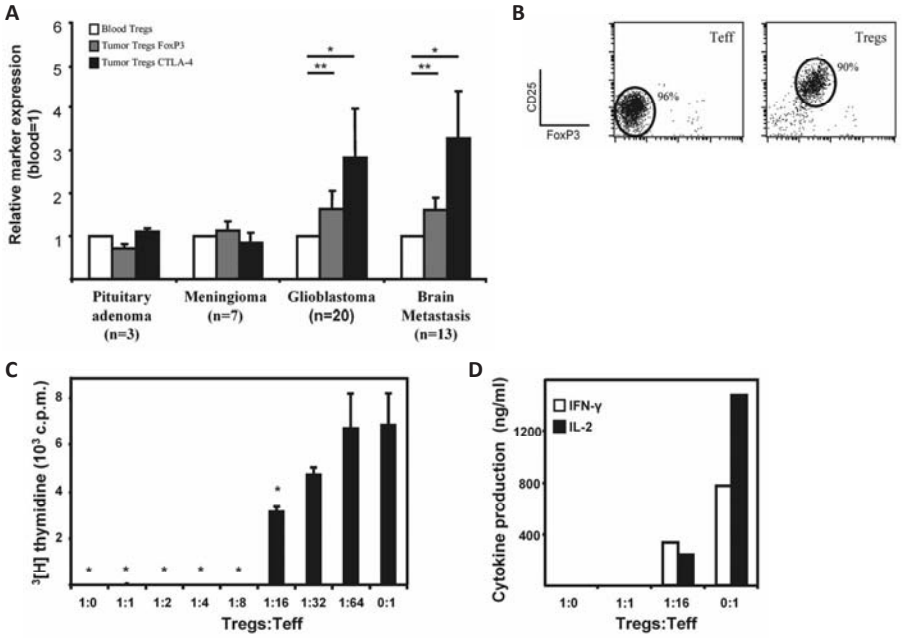
Brain metastasis (kidney) Brain metastasis (lung)

Immunohistochemistry. FoxP3 positive lymphocytes in paraffin-embedded brain tumors (236A/E7 antibody). BV=blood vessel.

Tregs infiltrated in malignant tumors are highly activated

Tregs infiltrated into high-grade brain tumors expressed significantly higher levels of the Treg activation markers FoxP3 and CTLA-4 (figure 4a). On average, Tregs in glioblastomas expressed 1.7 times as much FoxP3 and 2.8 times as much CTLA-4 than the Tregs in the blood of these patients ($p<0.01$). Tregs in brain metastases expressed 1.6 times as much FoxP3 and 3.3 times as much CTLA-4 compared to the Tregs in the blood of these patients ($p<0.001$). Tregs in the WHO-grade I brain tumors did not express increased levels of either FoxP3 or CTLA-4. The increase of CTLA-4 expression was caused by both an increase in intracellular CTLA-4 expression and cell-surface CTLA-4 expression (data not shown). These data indicate that Tregs present within high-grade gliomas and brain metastases are highly activated.

Figure 4



Tregs in high-grade brain tumors have an activated phenotype and strongly suppress proliferation of TILs. (a) FoxP3 and CTLA-4 expression is significantly increased in tumor infiltrating Tregs compared to peripheral blood Tregs in patients with glioblastomas and patients with brain metastases (*P<0.01; **P<0.001 paired Student's t-test). (b) Purified brain tumor derived $CD4^+FoxP3^-CD25^{low}CD127^{high}$ (effector T cells) and $CD4^+FoxP3^+CD25^{high}CD127^{low}$ (Tregs) were titrated in a suppression assay. (c) Proliferation was assessed by ^{3}[H]thymidine incorporation (*P<0.001 Student's t-test) and (d) cytokines were analyzed in supernatants. One representative experiment out of 3 is shown.

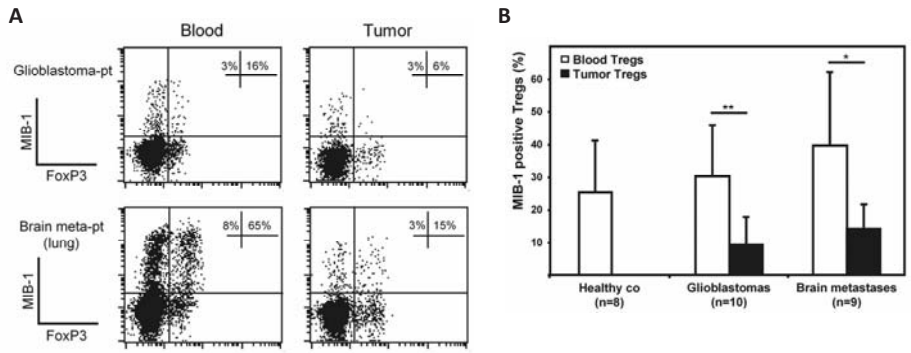
Intratumoral Tregs suppress lymphocyte proliferation

To test the suppressive capacity of intratumoral Tregs, TILs were sorted to obtain a $CD4^+CD25^{high}CD127^{low}$-fraction (>90% Tregs) and a $CD4^+CD25^{low}CD127^{high}$-fraction (>95% effector T cells) (figure 4b). The suppressive capacity of the intratumoral Tregs was analyzed in a titration experiment by incubating the cells with autologous intratumoral effector T cells, directly ex-vivo (n=3). As shown in figure 4c, the brain Tregs strongly suppress the proliferation of the autologous TILs (P<0.001). Already 1 Treg has a suppressive effect when incubated with 16 effector T cells. The decrease of effector T cell proliferation coincides with a strong decrease in the pro-inflammatory cytokines IFN-γ and IL-2 (figure 4d).

Proliferation of Tregs in-vivo

A key question is whether the Treg accumulation is due to specific tumor-recruitment or Treg expansion in the tumor microenvironment. Animal models have shown that Tregs can proliferate fast and that Treg turnover in-vivo is short.[21,22] We analyzed Treg-proliferation in-vivo by measuring the expression of proliferation marker MIB-1 (Molecular Immune Borstel-1) using the Ki-67 antibody.[23] In both patients with glioblastomas and in patients with brain metastases we detected a strong and significant decrease of MIB-1 expressing Tregs in the tumor compared to the Tregs in the blood (figure 5a and b, P<0.05). These data imply that the capacity of Tregs to proliferate declines as they infiltrate the tumor. The high percentage of MIB-1 positive Tregs in the blood of healthy controls and patients (range 11-65%) suggests a high turnover of these cells in-vivo.

Figure 5

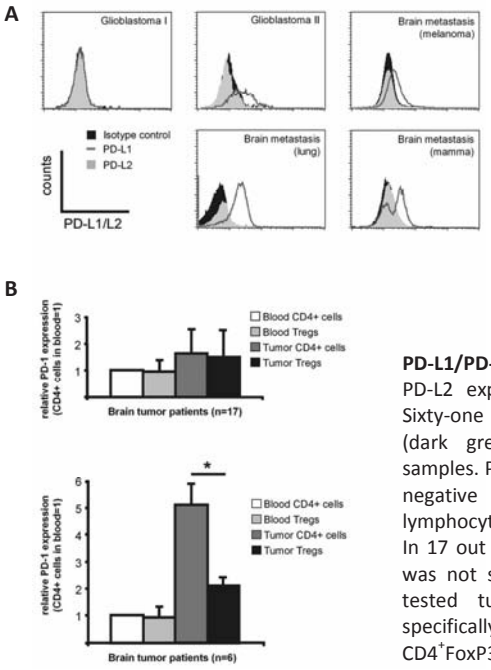


Treg proliferation in-vivo. (a) The percentages of MIB-1 (Ki-67 antibody) positive Tregs (of total $CD4^+FoxP3^+$) and MIB-1 positive $CD4^+$ cells (of total $CD4^+FoxP3^-$) are indicated in the top right quadrants of the dot-plots. Two representative patients are shown. (b) Overall MIB-1 expression on blood- and intratumoral Tregs of healthy controls, patients with glioblastomas and patients with brain metastases. The percentage of MIB-1 positive Tregs significantly decreases in the tumor (*P<0.05; **P<0.01 paired Student's t-test).

Contribution of PD-L1/PD-1 pathway in brain tumor immune evasion

Expression of programmed death ligand-1 (PD-L1) on tumors plays an important role in immune evasion due to interaction of PD-L1 with the inhibitory receptor PD-1 (PD-1) that is expressed on activated lymphocytes.[24] Sixty-one percent of the brain tumor samples in our cohort expressed PD-L1, the expression varied strongly between the tumors (figure 6a). None of the tested WHO-grade I brain tumors express PD-L1. In 17 out of 23 patients analyzed, the PD-1 expression on both tumor infiltrating $CD4^+$ cells and Tregs was low or negative (figure 6b). In 6 patients, PD-1 expression was detected on intratumoral $CD4^+$-effector T cells. The expression was significantly higher than the PD-1 on the intratumoral Tregs of these patients (figure 6c, P=0.005). PD-1 cell surface expression in blood lymphocytes was low or absent in all patients tested. These data indicate that the negative survival signal of PD-L1/PD1 pathway may represent another immune suppressive mechanism employed by malignant brain tumors.

Figure 6



PD-L1/PD-1 expression in brain tumors. (a) PD-L1 and PD-L2 expression in 5 representative brain tumors. Sixty-one % of the tumor samples expressed PD-L1 (dark grey line), which varied strongly between samples. PD-L2 expression (grey shaded histogram) was negative in all tested tumors (n=23). (b) Blood lymphocytes do not express PD-1 on their cell-surface. In 17 out of 23 tested tumors, PD-1 expression in TILs was not significantly upregulated. (c) In 6 out of 23 tested tumors, PD-1 cell-surface expression was specifically upregulated on tumor infiltrating $CD4^+FoxP3^-$ cells (*P=0.005 paired Student's t-test).

Discussion

The brain is a specialized immune site and represents a unique microenvironment to study immune cell/tumor interactions. Using ultrasonic aspirated brain tumor material as a bio-source we show that activated Tregs accumulate specifically in high-grade human brain tumors and brain metastases. These intratumoral Tregs acquire a fully activated phenotype and strongly suppress anti-tumor immune responses. A second important immune-regulatory pathway in brain tumors occurs via the PD-L1/PD-1 pathway.

Studying the tumor microenvironment is often hampered by the limited availability of fresh tumor material. We demonstrate that tumor cells and lymphocytes in ultrasonic aspirated material from brain tumors are viable and functional. Moreover, we provide direct evidence that this material resembles tumor biopsies in multiple aspects. In addition to studying the interplay between tumor cells and immune cells, ultrasonic aspirated brain tumor may represent a valuable bio-source for glioma cancer stem-cells. Furthermore, the tumor cells could be applied in the clinic to generate tumor lysate for immuno-therapeutic interventions.[25,26]

In peripheral blood, Tregs represent 5-10% of the $CD4^+$ T cell population. Tregs induce immune tolerance by suppressing host immune responses and thus play a critical role in preventing autoimmune disease.[27] However, Tregs also inhibit effective anti-tumor immune responses.[28] In a mouse glioma model, reversible specific depletion of Tregs significantly increased survival indicating the relevance of Tregs in glioblastomas.[15,17] Recent studies demonstrate that the presence of Tregs in various cancer types is correlated with a poor prognosis.[29] Glioblastoma is the only human brain tumor in which Tregs have been studied. Two independent studies confirm that Tregs can infiltrate malignant gliomas. However, the data on the Treg-fractions in the blood and tumor of these patients are contradictory.[30,31]

Studying Tregs in humans is complicated by the discovery that human activated non-regulatory $CD4^+$ T cells transiently express both CD25 and FoxP3.[32,33] Recent studies have demonstrated that downregulation of the IL-7 receptor (CD127) on Tregs distinguishes Tregs from activated T cells.[34,35] We therefore defined the Tregs in our study as $CD4^+FoxP3^+CD25^{high}CD127^{low}$ cells. Massive Treg infiltration in aggressive human primary brain tumors such as glioblastomas, and in metastatic brain tumors was readily observed. In contrast, Tregs did not accumulate in WHO-grade I meningiomas and pituitary adenomas. Overall, accumulation of Tregs in brain tumors is positively correlated with the WHO malignancy-grade of the tumor, except for WHO-grade I subependymal giant-cell astrocytomas. The reason for this is unclear and warrants further research. Currently, we determine the expression profiles of SEGAs and other WHO-grade I brain tumors using microarrays to define the genes responsible for Treg recruitment. Our immunohistochemical data show that brain tumor infiltrating Tregs are mainly localized perivascularly, a smaller fraction of Tregs infiltrate deep into the tumor, always in close proximity to other lymphocytes. This allows the Tregs to exert their contact-dependent suppression in-vivo.[36] Compared to healthy controls, no increase of Tregs in peripheral blood of patients with primary brain tumors was found. Only in case of brain metastases a systemic increase in Tregs could be demonstrated in the blood. Relative to blood-derived Tregs, Tregs isolated

from malignant brain tumors had a significantly increased expression of CTLA-4 and FoxP3, both of which are considered as activation markers that correlate with active immune suppression.[37,38] Indeed, the intratumoral Tregs strongly suppress the proliferation of autologous tumor derived lymphocytes directly ex-vivo, which suggests that patients can benefit from tumor-specific elimination of Tregs. We recently reported that in a murine glioma model, the immunosuppressive tumor environment can most efficiently be counteracted with Treg depletion in combination with active immunotherapy.[16] Depletion of human Tregs by targeting CD25 or FoxP3 might be an option,[39-41] although it should be noted that activated human effector T cells express significant amounts of CD25 and FoxP3.

To address the key-question whether the accumulation of Tregs is caused by an increased recruitment of Tregs to high-grade brain tumors or an increased proliferation of Tregs in the tumor we measured the proliferation marker MIB-1. MIB-1 expression in peripheral blood Tregs of both patients and healthy controls is very high compared to $CD4^{+}FoxP3^{-}$ T cells, as previously reported for mice.[22] In all tested patients, a sharp decrease of MIB-1 expression by intratumoral Tregs relative to peripheral blood Tregs was observed. Overall, these data imply that the specific accumulation of Tregs in high-grade brain tumors is not primarily due to Treg proliferation at the tumor site and are in line with recent mouse studies showing that actively proliferating Tregs do not suppress[42,43] and that tumors, including gliomas,[14] can secrete chemokines to recruit Tregs.[44-46] Initial MRI data imply that Treg accumulation can not be simply explained by the size of the brain tumor; most WHO-grade I tumors are large and harbour few Tregs. Possibly, structural and functional abnormalities in the vascular microenvironment in high grade brain tumors, causing loss of blood-brain barrier function,[47] do influence Treg infiltration. Blood-brain barrier integrity can be imaged using [18F]-fluoro-deoxy-L-fluorothymidine PET-scans. We currently analyse these scans to correlate lymphocyte infiltration with blood-brain barrier integrity. In addition, the brain tumor microenvironment may particularly favour Treg survival and function as glioblastomas can produce high levels of TGF-β which is known to be beneficial for Treg survival and the maintenance of its immune suppressive function.[48,49]

Recently, it was shown that PD-L1 is expressed on glial tumor cells.[12] We confirmed this observation and now show that PD-L1 is also expressed in various brain metastases. Interestingly, we observed in six high-grade brain tumors, that the inhibitory receptor PD-1 is expressed several fold higher on the cell surface of tumor infiltrating effector lymphocytes compared to the intratumoral Tregs. The PD-L1 expressed on brain tumor cells will therefore preferentially interact with the effector T cell population and may provide another selective survival advantage for Tregs in the tumor microenvironment.[24] Our data show that the PD-L1/PD-1 pathway appears to be important in a subset of brain tumor patients. Further research is needed to determine the basis of the variable expression of PD-L1 on tumor cells and the PD-1 expression on intratumoral Tregs between different patients.

In conclusion, we show that viable cell suspensions can be made from fresh ultrasonic aspirated tumor material. Using ultrasonic aspirated brain tumor material as a bio-source we show that activated Tregs specifically accumulate in high-grade human

brain tumors. The tumor-infiltrating Tregs are potent suppressors directly ex-vivo and thus hamper immune responses against the brain tumor. Abundant expression of PD-L1/PD-1 within the microenvironment of a selection of the brain tumors implies that this pathway represents a second immune-regulatory circuit active in brain tumors. These findings underscore the importance of controlling immune suppressive mechanisms as part of active immunotherapy protocols for the treatment of high-grade human brain tumors.

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Chapter 10

Summary and Discussion

Introduction

Cellular immunotherapy using dendritic cells (DCs) is an appealing approach to treat a child with cancer as it avoids the distressing long-term toxicities associated with conventional chemotherapy and radiation therapy.[1-3] DCs are professional antigen presenting cells that play a crucial role in both the initiation and modulation of cellular immune responses. Our increased understanding of DC biology and the possibility to obtain large numbers of DCs *in vitro* from isolated monocytes has boosted the use of DCs in tumor immunotherapy.[4-6]

Most of the DC-based vaccination trials have been conducted in adults. To date, three groups report promising clinical experiences using DCs to treat pediatric patients with solid tumors.[7-9] In all three studies, patients are treated with DCs that are pulsed with either whole tumor lysate or whole tumor RNA. The main advantages of using whole tumor is that 1) multiple tumor epitopes can be processed and presented by the DC which reduces the possibility of immune escape, 2) the tumor-epitopes do not necessarily have to be characterized and 3) it expands the clinical application as there is no MHC-restriction for patient inclusion.[10] A concern relevant to the use of whole tumor cell-derived material is that the generated immune response might not be 100% tumor-specific and therefore may cause autoimmune disease. Specific immune monitoring is also more difficult since the epitopes involved are unknown.[11]

Data presented in this thesis focus on DC culture in pediatric cancer patients and on the identification of antigens expressed on pediatric solid tumors. We translated our findings into a clinical feasibility trial using peptide pulsed autologous monocyte-derived DCs for the treatment of children with high risk solid tumors. This thesis further shows that regulatory T cells (Tregs) play an important role in the immune-regulation of tumors. The presence of Tregs in tumors may therefore have strong implications on the design of future immunotherapeutic trials. This chapter is an integrated discussion of these topics.

Results in this thesis

Dendritic cell culture for active immuno-therapy

Since the DC population in the peripheral blood is scarce, several techniques have been developed to generate DC from their precursors. In **chapter 1** we review how DCs can be cultured from $CD14^+$ monocytes. Numerous cytokine combinations have been reported to drive DC differentiation from their precursors, resulting in subtle though important differences between the generated DCs that determine the quality of the immune responses.[12] IL-12 and IFN-α produced by DC polarizes the T-cell response towards type 1 immunity, which is desirable for cancer immunotherapy. Since the DCs are used for in vivo application, the protocols for DC generation are adapted to good manufacturing practice guidelines to ensure safe administration to patients.

Several murine and human studies demonstrate that it is possible to induce specific anti-tumor immune responses and even tumor regression by injecting DCs pulsed with tumor lysates or tumor-specific peptides.[13-16] The immunizing ability of DCs in vivo is critically influenced by their maturation state and their capacity to migrate towards lymphoid tissue. Recent reports show that compared to immature DCs, mature DCs have a higher potency to migrate and induce specific immune responses.[17-21] Two phase I/II studies have described the use of semi-mature DCs in anti-tumor vaccines in pediatric cancer patients. These two studies reported promising results with both immunological and clinical responses.[7,8] Vaccine efficacy could be further optimized by using fully mature DCs since it is shown that maturation is a prerequisite for inducing immune responses in adult cancer patients.[17,21] In **chapter 2** we show that ex vivo generated, clinically grade, monocyte derived DCs from pediatric cancer patients can be generated in numbers sufficient for DC vaccination trials. Upon cytokine stimulation the DCs highly up-regulate expression of maturation markers. The mature DCs are six times more potent in inducing T cell proliferation compared to immature DCs. Furthermore, mature DCs, but not immature DCs, express the chemokine receptor CCR7 and have the capacity to migrate in vitro. These data indicate that mature DCs can be generated ex vivo to further optimize DC-vaccination trials in pediatric cancer patients.

T cell defined tumor antigens on pediatric solid tumors

In **chapter 3** we discuss the current status of cancer immunotherapy in children. Most immunotherapeutic therapies are still experimental and focus on adult patients. However, the first immunotherapeutic trials for pediatric cancer patients have been published and more are ongoing. We further describe the results of a literature study of tumor-specific antigens expressed on pediatric solid tumors that are recognized by T cells. Despite the paucity of relevant literature on this topic, we conclude that for most pediatric solid tumor types T cell defined antigens are reported.

One of the most widely used immune targets in clinical trials are the antigens encoded by cancer-germline genes (CGGs), such as MAGE and NY-ESO-1. These genes are expressed in different types of human tumors. They are not expressed in normal adult tissues with the exception of male germline which do not express HLA molecules and

therefore cannot present antigenic peptides to T cells.[22] For this reason the antigenic peptides encoded by CGGs are strictly tumor-specific. In addition to that, the high immunogenicity of the antigens, the availability of numerous clinical grade peptides and the availability of CGG-specific monitoring tools make these antigens ideal target antigens. In **chapter 4** we analysed CGG expression in a large panel of pediatric solid extra-cranial tumors. We show that CGGs are highly expressed in all osteosarcomas and in 80% of the neuroblastomas.

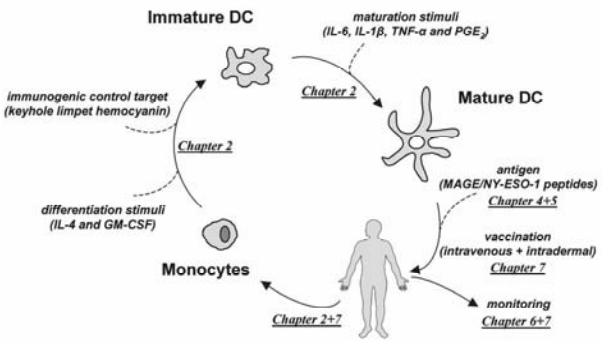
Brain tumors are the second most common malignancy in children, accounting for 20% of all pediatric tumors. It is the most common cause of cancer-related death in children.[23] Especially patients with a WHO grade IV tumor, such as a glioblastoma, have an extremely poor prognosis despite multimodal therapy. For these high grade brain tumors novel treatment modalities are urgently needed. For long the brain has been regarded as an immune-privileged site based on the presence of the blood-brain-barrier, lack of conventional lymphatics, and low immune cell trafficking. This is replaced by the idea that the brain is a specialized immune site where lymphocyte homing and immune response induction are regulated in a unique way.[24-26] Progress in our understanding of immune responses against brain tumors have already led to novel clinical applications[27,28], also for children with brain tumors.[8,29] In **chapter 5** we show that CGGs are expressed in approximately 30% of the pediatric brain tumors. CGG-encoded antigens are therefore suitable targets in a very selected group of pediatric patients with a brain tumor. Interestingly, glioblastomas from adult patients expressed CGGs more often and at significantly higher levels compared to pediatric glioblastomas. This observation is in line with the notion that pediatric and adult glioblastomas develop along different genetic pathways.[30,31]

Translational research

Since both overexpressed antigens and differentiation antigens are expressed at low levels in normal tissues, selecting these antigens as immuno-target may induce autoimmunity. However, since a minimal level of antigen expression is required for T-cell recognition, it is possible that many of the epitopes processed and potentially presented by normal tissues are below this threshold level.[32,33] Other factors that determine whether or not auto-immunity occurs are the number of antigen-specific T cells, their homing properties, T cell receptor affinity and cytotoxic capacities. In **chapter 6** we show that melanoma patients can develop vitiligo after immunotherapy using DCs loaded with tyrosinase and gp100 peptides. Tyrosinase and gp100 are differentiation antigens that are shared by both melanocytes and melanoma cells. We show that the occurrence of this auto-immune disease is indeed directly linked to the immuno-therapeutic intervention since we identified vaccine-specific CTLs inside these vitiligo lesions. In our cohort of patients, vaccine-induced vitiligo correlates with a favorable outcome which supports the hypothesis that vitiligo is a marker of immunity against melanoma cells. These findings stress that in an effective immunotherapeutic trial, the target antigen must either be specifically expressed on tumor cells or the normal tissue expressing the antigen must be dispensable, to avoid serious autoimmune toxicity.

Based on our experience with DC vaccinations in melanoma patients and our pre-clinical data regarding DC culture in pediatric patients and antigen expression on pediatric solid tumors, we introduced DC-immunotherapy for pediatric patients with high risk solid tumors (figure 1). In **chapter 7** we describe a clinical feasibility trial in which pediatric patients with high risk solid tumors are vaccinated with mature, monocyte-derived DCs loaded with tumor specific peptides. In this chapter we further describe the inclusion, treatment and follow-up of the first pediatric patient included in our study. We demonstrate that it is feasible to produce mature monocyte-derived DCs pulsed with MAGE-A1 peptides for vaccination in a pediatric patient with high risk osteosarcoma. No toxicity was observed during treatment and an antibody response against the control protein keyhole limpet hemocyanin (KLH) was detected in the serum of the patient. However, no MAGE-A1 specific T cells were detected and the patient had ongoing disease progression.

Figure 1



DC vaccination protocol in pediatric cancer patients. Monocytes isolated from peripheral blood are differentiated with GM-CSF and IL-4 into immature DCs, loaded with KLH and further matured using pro-inflammatory cytokines. Mature DCs are pulsed with tumor specific peptides derived from cancer germline genes and administered intravenously and intradermally. Chapters refer to chapters in this thesis for detailed information.

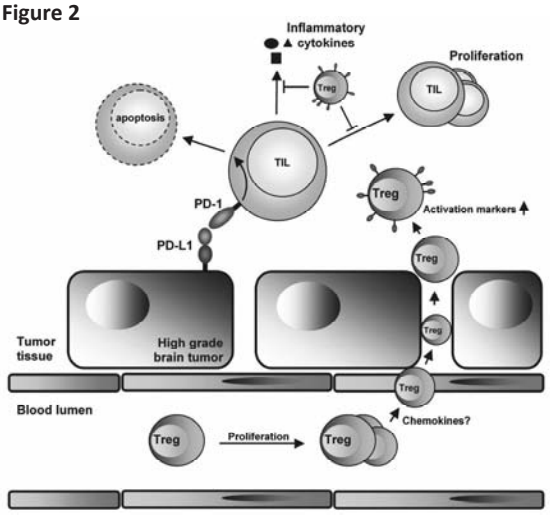
Immune regulation in the tumor microenvironment

In our ongoing clinical trials, we observed a direct correlation between the presence of DC vaccine-induced T cells and a positive clinical outcome.[34] However, some melanoma patients have progressive disease despite the presence of tumor infiltrating vaccine-induced melanoma-specific T cells. This is intriguing since only patients with melanomas that abundantly express the antigens are included in our studies. This suggests that tumor cells can acquire properties that allow them to evade immune attack. Four different mechanism have been described for this 'tumor immune-escape': evasion of immune-recognition, activation of negative co-stimulatory signals, secretion of immuno-suppressive factors and recruitment of regulatory T cells (Tregs).[35] Tregs have the potent ability to suppress host immune responses, thus

preventing autoimmune diseases and transplant rejection.[36-38] Recent studies demonstrate that tumor cells can recruit these Treg cells to inhibit antitumor immunity in the tumor microenvironment, thus limiting the efficiency of cancer immunotherapy.[39] The detrimental effect of Tregs in anti-tumor immunity is emphasized by murine cancer models showing that Treg depletion with monoclonal antibodies lead to significantly increased anti-cancer immunity.[40-44] In **chapter 8** we provide an overview of the different suppressive mechanisms employed by Tregs. We further describe that the expansion and the suppressive capacities of Tregs are influenced by Toll-like receptor (TLR)-ligands that signal via TLRs expressed on Tregs. Understanding the functional control of Tregs, including the role of TLR signaling, may offer new opportunities to shift the immune-balance in the tumor microenvironment from tolerance to immunity.

The brain is a specialized immune site where lymphocyte homing and immune response induction are tightly regulated. Therefore the brain represents a unique environment to study immune cell/tumor interactions.[24-26] Brain tumor infiltrating lymphocytes provide evidence that, similar to extra-cranial tumors, the immune system is naturally involved in the immunosurveillance of brain tumors.[27,45,46] High grade brain tumors, such as glioblastomas, can escape immune regulation by secretion of immunosuppressive cytokines,[47] activation of negative regulatory pathways in lymphocytes,[48,49] evasion of immune recognition[50] and recruitment of Tregs.[51] We recently demonstrated the relative importance of Tregs in this immunosuppressive network. In an experimental mouse glioma model we show a dramatic influx of Tregs[41] and demonstrate that temporal Treg depletion markedly augmented anti-tumor immunity.[42] These data strengthen the view that Tregs create a tolerogenic environment that hampers anti tumor-immunity.

Figure 2



Immune regulation in high grade brain tumors. Tregs, recruited from peripheral blood, accumulate in high grade brain tumors. Intratumoral Tregs have a reduced capacity to proliferate and acquire a fully activated phenotype. The intratumoral Tregs strongly suppress proliferation and cytokine production of tumor infiltrating effector lymphocytes. Tumor cells further express programmed cell death ligand-1 (PD-L1) which can interact with the programmed cell death receptor-1 (PD-1) on effector T cells. The negative signal conveyed by this pathway to the effector T cell can induce apoptosis or impaired proliferation and cytokine secretion.

In **chapter 9** we investigated the role of Tregs in a variety of human brain tumors. For functional studies on the tumor microenvironment relatively large volumes of freshly resected brain tumor are essential. To address this issue we developed a new method to isolate immune cells from ultrasonically resected brain tumor material. Most brain tumors are partially resected by neurosurgeons using an ultrasonic aspirator. Since this material is thought to be unsuitable for pathology, it is discarded. We demonstrate that tumor cells and lymphocytes in ultrasonic aspirated brain tumors are viable and functional. Using ultrasonic aspirated brain tumor material as a bio-source we show that activated Tregs accumulate specifically in high-grade human brain tumors and brain metastases. These intratumoral Tregs acquire a fully activated phenotype and strongly suppress anti-tumor immune responses. Next to these Tregs we describe a second important immune-regulatory pathway in high grade brain tumors which occurs via the PD-L1/PD-1 pathway (figure 2). These findings underscore the importance of controlling immune suppressive mechanisms as part of active immunotherapy protocols for the treatment of high-grade human brain tumors.

Future directions and challenges

Although clinical benefit of DC vaccinations for most cancer patients is limited to short disease stabilisation, some clinical responses to the vaccine are complete, durable and apparently curative. These sporadic successes combined with encouraging results in animal studies motivate tumor immunologists to further optimize clinical trials of cancer immunotherapy.[6]

The anti-tumor immune response

An important but not fully resolved issue is what kind of adaptive immune response is best suited to eradicate established tumors. Animal studies indicate a pivotal role for long lasting $CD8^+$ CTLs and T-helper 1 (Th1) $CD4^+$ cells. Therefore, it is not surprising that current efforts in developing cancer vaccines focus mainly on how to stimulate potent CTL responses. The Th1 subset of $CD4^+$ T cells is essential for the persistence of $CD8^+$ CTLs.[52,53] A long lasting immune response might be more important than a strong yet transient response in the setting of a chronic disease like cancer. In addition, $CD4^+$ T cells induce MHC class I upregulation on tumor cells which sensitizes tumor cells to CTL-induced lysis.[54] The importance of $CD4^+$ T cells is further demonstrated in murine studies, which have shown that $CD4^+$ T cells can eradicate tumor in the absence of $CD8^+$ T cells.[55] However, animal studies show that humoral immunity can also contribute in various ways to tumor eradication[56,57] and indicates that an exclusive focus of strategies to stimulate $CD8^+$ CTL responses is not justified.

Dendritic cell subset and maturation

DCs originate from $CD34^+$ bone marrow stem cells differentiating into either myeloid or lymphoid precursors. These precursors subsequently differentiate into Langerhans cells, interstitial DCs or plasmacytoid DCs.[58] All subtypes have their own phenotype and specialized function.[12] The discovery that GM-CSF and IL-4 can promote the differentiation of monocytes into immature DCs created strong progress in both the understanding of DC biology and their use in pioneering clinical trials.[59-61] Monocyte-derived DCs have the plasticity to differentiate into various types of DC after encounter with different cytokines and maturation factors. These distinct DC subsets influence lymphocyte differentiation. Therefore, both the type of DC subset and the activation signals to which DCs are exposed are important for polarization of T cells. The 'gold standard' method of inducing DC maturation with pro-inflammatory cytokines[62] needs to be examined in parallel to other stimuli. For example, Toll-like receptor (TLR) ligands can be used to mimic the in vivo pathogen-induced maturation of DCs. DCs express a broad repertoire of TLRs through which they sense microbes and initiate adaptive immune responses. Interestingly, triggering different TLRs causes synergistic DC activation.[63] Future research must identify the 'combinatorial code' that gives rise to enhanced T helper type 1-polarizing DCs with high migratory capacity. Recently, animal studies have also focused on administration of TLR ligands in vivo. Preliminary data indicate this boosts DC vaccination, not only by their effect on the DCs used for immunization, but also by activation/inhibition of other cells of the immune system or by a direct effect of the TLR ligands on the tumor.[64,65] With the recent application of RNA technology, DC biology can be further optimized to enhance immunological and

clinical responses.[66] In the near future, ex vivo cultured DCs could be equipped with enhanced expression of chemokine receptors to locate lymph nodes, silenced apoptotic pathways to increase the DC lifespan and upregulated production of pro-inflammatory cytokines to activate tumor specific T cells.

Antigen loading

Loading MHC class I and class II molecules at the cell surface of DCs with peptides derived from defined tumor associated antigens is a widely used strategy for DC-based vaccination. The main advantage of using a defined peptide is to generate an immune response that is very specific for that epitope. This reduces the chance of autoimmunity and facilitates immuno-monitoring. The use of peptides has however several limitations, such as the limited number of well-characterized tumor-associated antigens/peptides, the rapid turnover of exogenous peptides, the MHC restriction of the peptides limits patient inclusion and the induction of a restricted repertoire of T-cell clones thereby limiting the ability of the immune system to control tumor-antigen selection[10]. It is further hypothesized that naturally processed epitopes induce T cells that are superior in epitope recognition in vivo.[67] In conclusion, loading the DCs with the antigen, instead of selected peptides, allows 'natural' processing of the epitopes and is expected to improve efficacy as well as the generation of a more diverse immune response. Strategies for this include loading DCs with recombinant proteins or exosomes,[68] transducing DCs with viral vectors,[69] transfecting DCs with RNA[70] or plasmid DNA, loading DCs with immune complexes[71] or targeting DCs with antibodies specific for DC cell-surface molecules. The latter also opens the possibility for in vivo targeting of antigens to DCs,[72] which could replace the laborious and expensive ex vivo culturing in the future. The challenge will be to create vectors that specifically target DCs in vivo, provide antigens and subsequently induce DC maturation. The opportunity to reach and activate DC subsets in their natural environment with these vectors might lead to more efficient DC migration and antigen presentation.

Which antigens to choose for immunotherapy?

The identification of MAGE-1, a melanoma-specific antigen that stimulates human T cells in vitro, proved that the human immune system can respond to tumor antigens.[73] This new awareness stimulated a productive effort to discover new tumor antigens. The result is a long list of tumor associated antigens that could serve as targets for immuno-therapy.[74] With so many tumor associated antigens, choosing the best antigen for a specific immunotherapy trial is a crucial and difficult issue. The choice depends on the individual needs for that study, such as immunogenicity of antigen, level of antigen expression, tumor specificity and availability of tools to monitor antigen-specific immune responses.[75] The level of antigen presentation at the cell surface depends on various factors such as level of gene expression encoding the antigen, efficiency of processing relevant antigenic peptides, level of HLA molecules at the cell surface and the stability of this HLA-peptide complex. For gene MAGE-A1, a threshold of approximately three mRNA molecules per cell is required for CTL recognition.[76] These calculations can only be made when quantitative PCR is performed, therefore a positive conventional PCR using many amplification cycles is

less informative for patient inclusion. However, antigen processing efficiency can vary greatly between different antigens and between different tumors,[77] and stresses the fact that gene expression does not guarantee presentation to T cells. The T cell stimulation assays remain the gold standard for the presentation of a candidate antigen, which is extremely laborious as it requires a tumor cell line and a T cell clone. For a few antigens, antigen presentation can be tested using soluble reagents that directly label HLA-peptide complexes at the cell surface, as tetramers do for specific TCR staining.[78-80] For an NY-ESO-1 peptide presented by HLA-A2 molecules, presentation was tested on different cancer cells and varies between 10-50 complexes per cell. This density was sufficient for CTL recognition.

For safety concern, ideally, the target antigens in clinical studies should only be expressed by the malignant cells and not by normal cells. If a target antigen specifically expressed on tumor cells is not available, the normal tissue expressing the antigen must be dispensable, to avoid serious autoimmune toxicity. Another method to avoid the risk of auto-immunity is to use antigens against which some cancer patients, without overt auto-immunity, spontaneously mount T cell responses. It is important to abide these principles for safety as more efficient vaccination modalities will become available in the future.

Finally, it is preferable to target combinations of antigens and antigens encoded by genes that are essential for tumor cell survival or growth as this decreases the chance of in vivo selection of antigen-negative tumor cells. Interestingly, an immune response targeted against a specific epitope can give rise to effective immune responses against other tumor specific epitopes.[81] This broadening of the immune response is a process referred to as epitope spreading. Several, not mutually exclusive, mechanisms may explain the phenomenon of epitope spreading. First, the generation of an effective anti-tumor immune response can release new antigens in apoptotic bodies that are released from killed tumor cells.[82,83] Second, it is also possible that the reduction in tumor burden caused by an effective vaccine relieves immunosuppression in the tumor microenvironment caused by inhibitory cytokines and Tregs, thereby contributing to a broadened anti-tumor immune response. Finally, it is hypothesized that anti-vaccine T cells that infiltrate a tumor serve as a spark that triggers the activation and subsequent proliferation of ineffective CTLs that were already present inside the tumor.[84]

Monitoring immune responses

Objective clinical responses induced by DC vaccination are preceded by effective anti-tumor immune responses[34]. In the absence of such a clinical response, monitoring the immunologic outcome is of crucial importance for the interpretation of study results. Reliable, reproducible and quantitative assays to evaluate the vaccine-induced immune response are essential to move from promising phase I and II clinical studies to decisive phase III studies. Standardization of these assays becomes increasingly important since new clinical trials are often multi-centre efforts. Characterization of vaccine-specific T cells is essential for assessing proof-of-principle of a new immunotherapeutic intervention. Vaccine-induced T cells can be quantified, functionality tested for cytotoxicity, T cell homing capacity and TCR clonality[11,85].

Peptide MHC tetramers can be used to identify and isolate vaccine-induced T cells, tetramers can be combined with other techniques to further profile the phenotype and function of vaccine-specific T cells in vitro[86]. Although peptide MHC tetramers are powerful tools for immuno-monitoring, they have limitations. They can only be used to detect immune responses to known antigens. Even immunizations with a single peptide can induce an immune response that is broadened to other epitopes, as explained earlier by the phenomenon of epitope-spreading. Monitoring the magnitude of this response is difficult because not all tumor antigens involved are known. Another important parameter that needs to be monitored in DC vaccination trials is DC migration in vivo, especially because the optimal route of DC administration is a still unresolved issue. In many clinical trials, DCs are administered intradermally and/or intravenously.[87,88] However, these routes of administration rely on the capacity of the injected DCs to migrate towards the lymph nodes. This problem can be circumvented by direct intranodal injection of DCs under ultrasonic guidance.[89] When DCs are labeled with a radioisotope or a superparamagnetic particle the efficiency of DCs migration after vaccination can be tracked in vivo.[90]

The development of the delayed type hypersensitivity reaction (DTH test) is an important monitoring tool that assesses the anti-tumor immune response in vivo. Antigens used for vaccination are injected intradermally into the patient, this attracts immune cells to the sensitization site. The extent of immune invasion is then a measure for the strength of the immune response induced by the vaccine. If a biopsy of the DTH-site is taken, the immune cells can be isolated for further phenotypic and functional characterization[34]. Since the microenvironment of the immune response plays a crucial role we foresee that immuno-monitoring in the future will focus on the in vivo situation. Accurate monitoring can only be done in the context of the tumor microenvironment and tumor draining lymph nodes.

Regulation of immune-suppression in the tumor microenvironment

Tumors and associated parenchymal cells can establish robust immune privilege, such that natural and vaccine-induced tumor-specific immunity can be completely blocked. Because immune surveillance mechanisms eliminate many pre-cancerous lesions[91], it is hypothesized that the tumors that survive to form mature malignancies have already acquired immune-evasive properties. Tumor immune-escape can be based on four mechanisms: evasion of immune-recognition, activation of negative co-stimulatory signals, secretion of immuno-suppressive factors and recruitment of immune-suppressive cells.[35] Importantly, the suppressor mechanisms are not limited to the tumor but also play a role, for example, in tumor draining lymph nodes.[92] The suppressive mechanisms apply to the initiation-, effector- and memory phase of the immune response. This multilevel local suppression might explain the limited clinical responses to cancer immunotherapies, even when they induce systemic immune responses. Elucidating the fundamental mechanisms by which tumors are shielded from effective immune responses may lead to strategies that create a tumor environment that favors immunity. Obviously, efforts must focus on a local disruption of immune-suppressive mechanisms as systemic, irreversible interventions will cause auto-immunity.

In this thesis we have shown that Tregs with a strong suppressive capacity accumulate in high grade brain tumors. The relative importance of Tregs in the immunosuppressive network of brain tumors is demonstrated in experimental mouse glioma models that show a dramatic influx of Tregs and temporal Treg depletion markedly augmented anti-tumor immunity.[41,42,93,94] Understanding the functional control of Tregs, including the role of TLR signaling, may offer new opportunities to regulate these cells. This, and the identification of specific targets on Tregs that allow their elimination from the tumor microenvironment, represent some of the major challenges towards the development of effective cancer immunotherapy.

Combining DC vaccination with other therapies

The therapies that can be combined with immuno-therapy can be divided into two groups: therapies that improve the vaccine itself and therapies that simultaneously debulk tumor cells. For the latter, any standard therapies such as surgery, local radiotherapy and chemotherapy prior to immunotherapy reduces tumor mass. This increases the effector lymphocyte:tumor cell ratio, and favors immune mediated tumor rejection.[95,96] Several of the cancer chemotherapeutics are however immunosuppressive, they are in fact used as immunosuppressants for the treatment of severe systemic autoimmune diseases.[97,98] Other chemotherapeutics may have effects that favor immunotherapy, through transient lympho-depletion, by the subversion of immunosuppressive mechanisms or through direct or indirect stimulatory effects on immune effectors. The other way around, vaccination against cancer-specific antigens can sensitize the tumor to subsequent chemotherapeutic treatment.[99]

Therapies that improve the vaccine itself either facilitate the biodistribution of the vaccine, provide help for the elicited T cells or antagonizes negative immune regulation in the tumor environment. Studies in mice show that pre-injection of TNF at the site of DC-vaccination improves the migration of the DCs to the draining lymph nodes.[100] Concomitant administration of other cytokines could improve DC-efficacy and support induced T cells.[101] Studies in mice have for example indicated that type I IFNs support T cells in vivo; this occurs either directly, by sustaining T-cell survival[102], or indirectly, by triggering antigen presenting cells to release IL-15 which in turn enhances T-cell growth.[103] As discussed earlier, tumor-immune escape mechanisms can have a dramatic effect on immuno-therapeutic interventions. A better understanding of the interactions between tumors and the immune system will lead to novel and more effective strategies to neutralize the immunosuppressive environment surrounding the tumor. It will be of critical importance to determine the effectiveness of combined strategies involving immunotherapy together with blockade of immune-inhibition in the tumor microenvironment.[104,105] In fact, progress has been made in this field by evaluating the effects of anti-tumor immunotherapeutic intervention combined with CTLA-4 block,[106,107] programmed cell death receptor-1 (PD-1) block,[108] or Treg depletion.[42] Early results of these combinatorial approaches that induce DC-mediated tumor antigen presentation and antagonize negative immune regulation have shown that significant tumor destruction without the induction of serious autoimmune disease is possible.

Immunoprevention of cancer

Viral infection can result in the malignant transformation of the host's infected cells. Examples of cancers associated with viruses are Kaposi's sarcoma (linked with human herpes virus 8), cervix carcinoma (human papilloma virus), various lymphomas (Epstein-Barr virus) and hepatocellular carcinoma (hepatitis B and C)[109]. It has already been demonstrated that prophylactic vaccinations against viral antigens such as those of hepatitis B virus and human papillomavirus lower the risk of hepatocellular carcinoma[110] and cervical cancer[111], respectively. Should similar vaccines against tumor antigens prove valuable, they could be used in a prophylactic setting in young adults who are genetically predisposed to cancer. For example for patients with mutations in the BRCA1 or BRCA2 genes (lifetime risk of 85% to develop breast- or ovarian cancer[112]) or mutations in mismatch repair genes (lifetime risk of 80% to develop colon- or endometrium cancer[113]). Tumor-challenges in immunized rodents and cancer-prone mice show that vaccines can prevent tumor onset and progression[114]. It is hypothesized that these prophylactic vaccines are effective in tumor prevention because the target is a small precancerous lesion. Therefore, most of the difficulties that are encountered by vaccines directed against established tumors do not apply.

Outlook

Immunotherapy against cancer using antigen-loaded DCs is a field of growing interest. Early experimental clinical trials have focused on adult patients and provided proof-of-principle. Trials in pediatric patients have been scarce due to small patient numbers and a paucity of tumor specific antigens identified on pediatric tumors. Meanwhile, the first immunotherapeutic trials for pediatric cancer patients have been published, and more are ongoing. Further optimization of parameters such as DC maturation, antigen selection, antigen loading and monitoring must facilitate this clinical endeavor. Efforts should also focus on the combination of DC vaccination with other therapies that reduce tumor load or neutralize the immunosuppressive micro-environment in tumor tissue.

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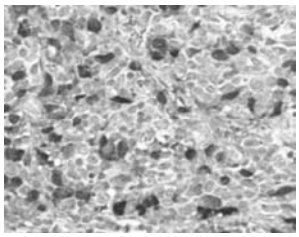
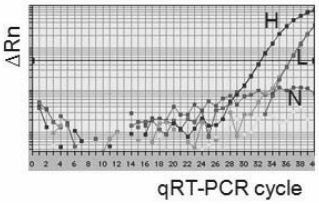
Color Figures

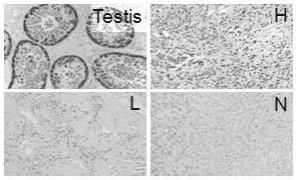
Chapter 3, Figure 2A



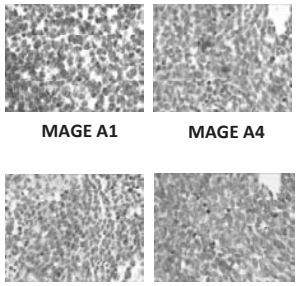
Chapter 5, Figure 3A



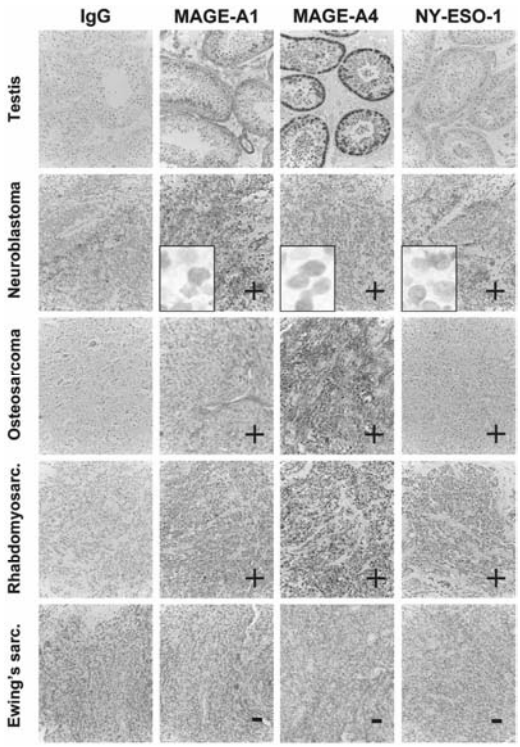
Chapter 5, Figure 3B



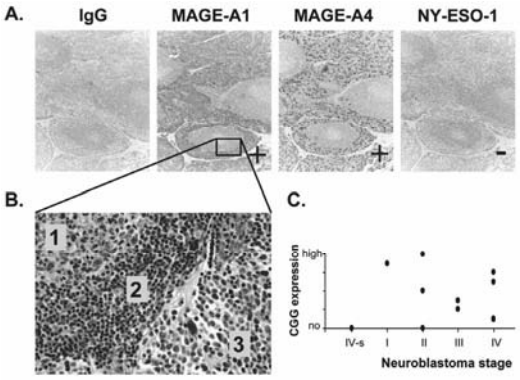
Chapter 5, Figure 3C



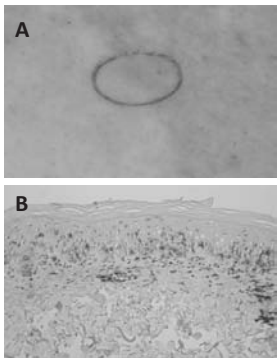
Chapter 4, Figure 3



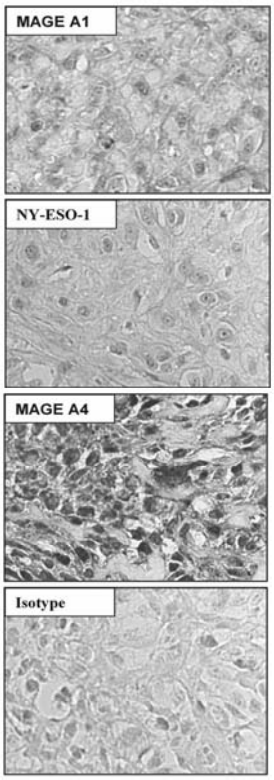
Chapter 4, Figure 4



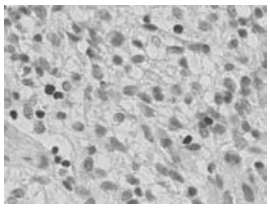
Chapter 6, Figure 1



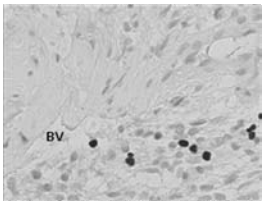
Chapter 7, Figure 2



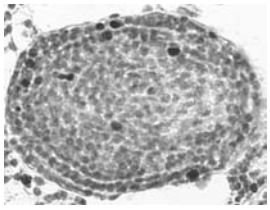
Chapter 9, Figure 3



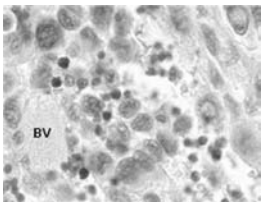
Glioblastoma (1)



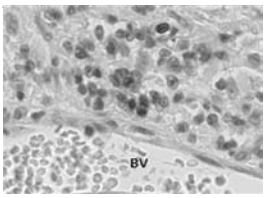
Glioblastoma (2)



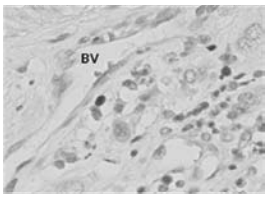
Brain metastasis (lymphoma)



Brain metastasis (breast)



Brain metastasis (kidney)



Brain metastasis (lung)

Nederlandse samenvatting

Inleiding

Achtergrond en doel van het onderzoek.

Per jaar wordt er in Nederland bij ongeveer 500 kinderen kanker vastgesteld. Voor kinderen tussen 1 en 18 jaar is kanker de belangrijkste natuurlijke doodsoorzaak. Kanker ontstaat door veranderingen in lichaamscellen, waardoor ze ongeremd kunnen gaan groeien. Chirurgie, chemotherapie en radiotherapie vormen samen de belangrijkste peilers voor de standaard behandelmethode van kanker. Ondanks de effectiviteit van de huidige therapieën, schieten zij bij een aantal patiënten tekort. Daarom is er onderzoek nodig naar nieuwe aanvullende behandelmethoden.

Dit proefschrift gaat over onderzoek naar immunotherapeutische strategieën voor de behandeling van kinderen met hoog risico, solide tumoren. Dit betekent dat, bij kinderen met kanker waarbij standaard therapieën tekort schieten, wordt getracht het eigen afweer systeem te stimuleren om kankercellen op te ruimen. In de verschillende hoofdstukken van dit proefschrift wordt uitgelegd wat het afweersysteem is en hoe immunotherapie werkt. Met zowel laboratorium onderzoek als klinische data tonen we aan dat het afweersysteem tegen specifieke structuren van kankercellen bij kinderen gericht kan worden. Ten slotte laten we zien dat kankercellen zich daartegen wapenen door te proberen het afweersysteem te ontduiken.

Het immuunsysteem

Het immuunsysteem beschermt het lichaam tegen ziekteverwekkers, zoals bacteriën, parasieten en virussen, maar ook tegen veranderde eigen cellen zoals kankercellen. Het immuunsysteem kan worden onderverdeeld in het adaptieve (verworven) immuunsysteem en het aspecifieke (aangeboren) immuunsysteem. Aangeboren immuniteit omvat barrières tegen besmetting zoals huid en de slijmdeklaag van de darm en de luchtwegen, die fysisch verhinderen dat ziekteverwekkers het lichaam binnendringen. Daarnaast kunnen bepaalde cellen en componenten van het aangeboren immuunsysteem snel gemobiliseerd worden om binnengedrongen ziekteverwekkers te herkennen en op te ruimen. Het adaptieve immuunsysteem treedt in werking na het aangeboren immuunsysteem en bestaat onder andere uit B- en T lymfocyten. Het adaptieve immuunsysteem heeft in tegenstelling tot het aangeboren immuunsysteem een geheugenfunctie. Voor activering van het adaptieve immuunsysteem worden ziekteverwekkers en diens bestanddelen die door het afweersysteem herkent worden, z.g. antigenen, opgenomen door antigeen-presenterende cellen. Daarna vervoeren de antigeen-presenterende cellen deze antigenen naar de lymfeklieren om daar de antigenen te presenteren aan de lymfocyten. De antigeen-presenterende cellen activeren T lymfocyten tot antigeen

specifieke 'killer'-cellen of zij instrueren B lymfocyten om specifieke antistoffen te produceren. Het zijn deze killercellen en antistoffen die vervolgens de ziekteverwekkers opruimen.

Het immuunsysteem en kanker

Ook kankercellen bevatten antigenen. Het immuunsysteem kan killercellen en antistoffen maken die moeten voorkomen dat kanker ontstaat of die ervoor zorgen dat kankercellen worden opgeruimd. Mensen bij wie het immuunsysteem niet goed functioneert hebben, naast een verhoogde kans op infecties, ook een verhoogd risico om kanker te krijgen. Bij immunotherapie tegen kanker wordt met een vaccin een afweerreactie opgewekt, die in staat is om kankercellen te vernietigen. In **hoofdstuk 1** beschrijven we dat dendritische cellen, dit zijn antigeen-presenterende cellen, buiten het lichaam van de patiënt gekweekt kunnen worden via voorlopercellen uit het bloed van een patiënt met kanker. Deze dendritische cellen worden in het laboratorium beladen met antigenen afkomstig van de kankercellen van de patiënt. Met een cocktail van rijpingsfactoren kunnen de dendritische cellen in het laboratorium verder geactiveerd worden. Vervolgens worden deze geactiveerde en beladen dendritische cellen ingespoten bij de patiënt, de zogenaamde vaccinatie. Indien de dendritische cellen de lymfeklieren bereiken, zullen zij de antigenen presenteren aan de daar aanwezige lymfocyten. Daarbij worden lymfocyten geactiveerd waardoor een specifieke afweerreactie wordt opgewekt tegen de antigenen afkomstig van kankercellen.

Wereldwijd zijn er met bovenstaande vaccinatie-methode patiënten met uiteenlopende vormen van kanker experimenteel behandeld. Met name de wijze waarop de dendritische cellen in het laboratorium geactiveerd en beladen worden heeft een sterke invloed op de uitkomst van de behandeling. Samenvattend kan gesteld worden dat de vaccinaties weinig bijwerkingen geven. Bij een aantal patiënten wordt na vaccinatie een specifieke afweerreactie tegen de kankercellen waargenomen en dit gaat gepaard met een langere overleving van deze patiënten. Echter, het percentage patiënten waarbij de kankercellen volledig en blijvend opgeruimd worden door de vaccinaties is laag.

Dit proefschrift

Dendritische cellen bij kinderen met kanker

In de studies beschreven in dit proefschrift is onderzocht of het mogelijk is om bovenstaande immunotherapie toe te passen bij kinderen met een hoogrisico kankergezwel. Theoretisch zouden juist kinderen baat kunnen hebben van de

vaccinaties vanwege de verhoogde effectiviteit van het immuunsysteem bij kinderen. In **hoofdstuk 2** laten we zien dat dendritische cellen in het laboratorium gekweekt kunnen worden uit voorlopercellen afkomstig van bloed van kinderen met kanker. De dendritische cellen kunnen verder geactiveerd worden waardoor ze efficiënt de lymfeklieren in het lichaam kunnen bereiken. De dendritische cellen kunnen bovendien efficiënt lymfocyten activeren voor het opwekken van een afweerreactie. Uit het bloed zijn voldoende dendritische cellen te kweken om te gebruiken voor de vaccinaties.

Antigenen op kankercellen afkomstig van kinderen
Om een afweerreactie specifiek te kunnen richten tegen kankercellen, is het nodig om te weten welke antigenen er voorkomen op de kankercellen. Bij de vormen van kanker die voorkomen bij volwassenen is daarover al veel onderzoek verricht, in tegenstelling tot bij kinderen. In **hoofdstuk 3** hebben we een literatuuronderzoek verricht naar antigenen op kankercellen afkomstig van kinderen. We bediscussiëren welke antigenen het beste gebruikt kunnen worden voor immunotherapie.

Een van de meest gebruikte antigenen voor immunotherapie bij volwassenen met kanker, zijn antigenen afkomstig van een familie van genen die we de Tumor Testis Antigenen (TTA) noemen. MAGE en NY-ESO-1 zijn de belangrijkste TTA-familieleden. Deze TTA zijn aanwezig in verschillende vormen van kanker bij volwassenen en kunnen veilig gebruikt worden om een afweerreactie tegen te starten. In **hoofdstuk 4** laten we zien dat deze TTA ook tot expressie komen in sommige kankergezwellen afkomstig van kinderen. TTA zijn met name aanwezig in botkanker en neuroblastomen. Deze laatste vorm van kanker komt alleen voor bij kinderen en ontstaat uit zenuwvezels.

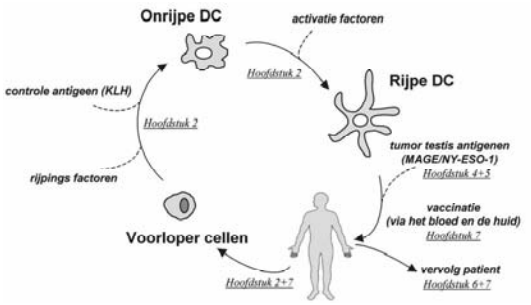
Hersentumoren zijn, na leukemie, de meest voorkomende vorm van kanker bij kinderen. Met name sommige vormen van hersentumoren, zoals glioblastomen, zijn zeer moeilijk te behandelen en hebben daardoor een slechte prognose. De bloedvaten van de hersenen zijn anders georganiseerd dan de bloedvaten elders in het lichaam. Ze beschermen de hersenen doordat ze minder doorlaatbaar zijn voor cellen en grote eiwitten, zij vormen de zogenaamde bloed-hersenbarrière. Er werd lang gedacht dat het afweersysteem geen toegang heeft tot de hersenen vanwege deze barrière. Onderzoek heeft uitgewezen dat lymfocyten en antistoffen wel kunnen doordringen in hersentumoren en patiënten met een hersentumor kunnen baat hebben bij immunotherapie. In **hoofdstuk 5** laten we zien dat in ongeveer 30% van de hersentumoren bij kinderen TTA aanwezig zijn. Deze antigenen kunnen dus slechts bij een selecte groep patiënten gebruikt worden als doelwit om de immunotherapie tegen te richten. TTA komen nauwelijks voor in glioblastomen die ontstaan bij kinderen. Dit

staat in scherpe tegenstelling tot onze bevindingen bij glioblastomen van volwassenen. Onze resultaten sluiten goed aan bij onderzoek van twee andere internationale groepen die ook al lieten zien dat glioblastomen van kinderen op sommige aspecten verschillen van glioblastomen die voorkomen bij volwassenen.

Klinisch onderzoek

Ons instituut heeft inmiddels ruime ervaring met dendritische cel vaccinaties bij volwassen patiënten met moedervlek kanker (melanoom). De dendritische cellen worden daarvoor beladen met antigenen die in hoge mate voorkomen op het melanoom, namelijk gp100 en tyrosinase. Echter, deze twee antigenen komen ook in lage concentraties voor op normale pigmentvormende cellen (melanocyten). Bij enkele patiënten zien we vitiligo (ontkleuring van de huid) ontstaan als bijwerking van deze immunotherapie. In **hoofdstuk 6** bewijzen we dat deze bijwerking een direct gevolg is van de vaccinaties. Bij analyse van een klein stukje van de ontkleurde huid tonen we aan dat de ontkleurde huid geïnfiltreerd wordt door T lymfocyten. Verdere analyse van deze T lymfocyten laat zien dat ze specifiek gericht zijn tegen gp100 en tyrosinase, de twee antigenen die verwerkt zijn in het vaccin. De groep van patiënten die vitiligo ontwikkelen na vaccinatie hebben gemiddeld een betere prognose dan de patiënten die geen vitiligo ontwikkelen na vaccinatie. Deze bevindingen ondersteunen de hypothese dat het ontwikkelen van vitiligo na vaccinatie een aanwijzing geeft dat er een afweerreactie plaatsvindt tegen het melanoom.

Figuur 1



Dendritische cel (afgekort als DC) vaccinatie protocol voor kinderen met een kankergezwel. Voorlopercellen uit het bloed worden in het lab gekweekt tot rijpe DCs met behulp van rijpings factoren en activatie factoren. De DCs worden beladen met het controle antigeen KLH en met kanker-specifieke tumor testis antigenen. Via het bloed en de huid worden de dendritische cellen teruggegeven aan de patiënt, de zogenaamde vaccinatie. In de figuur wordt verwezen naar de verschillende hoofdstukken in dit proefschrift voor meer gedetailleerde informatie.

In **hoofdstuk 7** beschrijven we een fase I/II klinische studie waarbij kinderen met een hoogrisico kankergezwel behandeld worden met dendritische cellen die beladen zijn met antigenen afkomstig van het kankergezwel (zie figuur 1). In dit hoofdstuk beschrijven we tevens de inclusie, behandeling en klinisch vervolg van het eerste kind dat behandeld is volgens dit nieuwe protocol. Deze patiënt had een onbehandelbaar osteosarcoom (botkanker) waarop het antigeen MAGE-A1 voorkwam. Uit voorlopercellen afkomstig van het bloed van de patiënt konden voldoende dendritische cellen gekweekt worden voor een volledige vaccinatie-therapie. De dendritische cellen werden vervolgens geactiveerd en beladen met het MAGE-A1 antigeen en een controle antigeen genaamd KLH. Er werden geen bijwerkingen van de therapie waargenomen. Er werd een afweerreactie tegen het controle antigeen KLH waargenomen na vaccinatie. Er kon echter geen specifieke afweerreactie worden waargenomen tegen het MAGE-A1 antigeen en de ziekteprogressie kon niet gestopt worden.

Kankercellen ontsnappen aan het immuunsysteem

Melanoompatiënten waarbij het lukt om met dendritische cel vaccinaties een kanker-specifieke afweerreactie te induceren, leven gemiddeld langer dan patiënten waarbij dit niet lukt. Toch hebben enkele melanoompatiënten progressie van hun ziekte ondanks de aanwezigheid van een specifieke afweerreactie tegen de kankercellen. Dit suggereert dat kankercellen eigenschappen kunnen aannemen waardoor ze aan het immuunsysteem kunnen ontsnappen, er zijn enkele mechanismen beschreven die dit verklaren. Kankercellen kunnen er bijvoorbeeld voor zorgen dat er minder antigenen op hun oppervlak voorkomen, waardoor ze als het ware 'onzichtbaar' worden voor het immuunsysteem. Kankercellen kunnen ook factoren uitscheiden die een remmende invloed hebben op het afweersysteem. Hierdoor wordt een milieu gecreëerd rondom het kankergezwel waarin het afweersysteem niet goed functioneert. Ten slotte kan een kankergezwel tevens regulerende T lymfocyten (Tregs) aantrekken. Tregs zijn speciale lymfocyten in het lichaam die ervoor zorgen dat een afweerreactie ook weer stopt indien nodig. Tevens zorgen Tregs ervoor dat het afweersysteem zich niet richt tegen gezonde weefsels. Tregs vormen dus feitelijk de 'rem' van het immuunsysteem. In **hoofdstuk 8** beschrijven we op welke manieren Tregs het immuunsysteem kunnen remmen. Kankercellen kunnen stoffen uitscheiden waardoor Tregs worden aangetrokken. Kankergezwellen waarin veel Tregs voorkomen groeien gemiddeld genomen agressiever doordat het afweersysteem in deze gezwellen niet goed functioneert. In onderzoek bij muizen is aangetoond dat een kankergezwel minder snel groeit, of zelfs verdwijnt, indien tijdelijk de Tregs uitgeschakeld worden.

In **hoofdstuk 9** onderzoeken we het functioneren van het immuunsysteem en de rol van Tregs in hersentumoren. Tijdens een operatie worden de meeste hersentumoren gedeeltelijk verwijderd door ze met behulp van hoogfrequent geluid los te trillen en weg te zuigen. Wij hebben een methode ontwikkeld om cellen van het afweersysteem uit dit weggezogen materiaal te isoleren. Hiermee wordt het mogelijk om de Tregs in hersentumoren te analyseren en functioneel te testen. We laten zien dat met name in agressieve, snel groeiende hersentumoren procentueel veel Tregs voorkomen. Deze Tregs zijn volledig geactiveerd en kunnen het immuunsysteem efficiënt onderdrukken. Immunotherapeutische behandeling van agressieve hersentumoren zal in de toekomst daarom wellicht gecombineerd moeten worden met een tijdelijke uitschakeling van de Tregs.

Conclusie

Voor aanvullende behandeling van hoogrisico kankergezwellen wordt in toenemende mate gebruik gemaakt van immunotherapie waarbij een afweerreactie wordt geïnduceerd tegen de kankercellen. Tot nu toe heeft dit onderzoek zich met name toegespitst op volwassenen met kanker. Voor toepassing hiervan bij kinderen is nog veel onderzoek nodig. In dit proefschrift beschrijven we dat het mogelijk is om kinderen met kanker te vaccineren met dendritische cellen waarbij een afweerreactie wordt geïnduceerd tegen bepaalde antigenen op het kankergezwel. Hierbij moeten echter nog veel parameters geoptimaliseerd worden die er enerzijds voor moeten zorgen dat een sterke afweerreactie zich specifiek richt tegen de kankercellen en anderzijds moet voorkomen dat de kankercellen aan deze afweerreactie kunnen ontsnappen. Voor een optimaal effect kan deze vorm van immunotherapie in de toekomst wellicht gecombineerd worden met standaard therapieën zoals chirurgie, chemotherapie en radiotherapie.

Dankwoord

Bij het uiteindelijke bundelen van dit boekje, realiseer ik mij pas hoeveel mensen hieraan hebben bijgedragen. Ik wil dan ook graag dit team van collega's, vrienden en familie bedanken voor alle adviezen, steun en vertrouwen. Bedankt, met name, voor het simpele feit dat ik de afgelopen 4 jaren elke dag met een brede lach van en naar mijn werk heb kunnen fietsen.

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I would like to thank the members of the manuscript committee Prof. dr. Theo de Witte, Prof. dr. Maarten Egeler and Prof. dr. Pierre Coulie. I am grateful for your time and effort to evaluate my manuscript and prepare for the thesis defense.

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Prof. dr. Pierre Coulie thanks for your kind invitation to conduct research at your department of the Ludwig Institute of Cancer Research in Brussels. Together with Francis and Madeleine we worked on the identification of novel immune targets on pediatric solid tumors. This alliance has resulted in nice publications I am very proud of.

Hoe een bijzondere patiënt of een gekke laboratorium meting kan leiden tot één experimentje, gevolgd door een ander experiment dat leidt tot een pilot-studie dat vloeiend overgaat in een klein project… en voordat de bazen echt beginnen te klagen over out of focus raken, gelukkig tot hele mooie artikelen. Bas en Arjan, super bedankt voor de leuke samenwerking en het kijkje in de keuken van respectievelijk de neuro-chirurgie en de antropogenetica.

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Hans, alias Zwelgje.

Curriculum Vitae & List of Publications

Curriculum Vitae

Joannes (Hans) Franciscus Maria Jacobs werd geboren op 29 juli 1977 te Weert. Hij behaalde in 1995 zijn atheneum diploma aan de Philips van Horne Scholengemeenschap te Weert. In 2000 behaalde hij zijn doctoraal biomedische wetenschappen aan de Radboud Universiteit Nijmegen. In 2001 ontving hij zijn doctoraal geneeskunde, gevolgd door zijn artsexamen in 2004. Deze studies volgde hij gedeeltelijk aan de *Kansas State University* (VS) het *Karolinska institute* (Zweden) en *Mukumu Hospital* (Kenia). Improvisatie-theater was tijdens zijn studie zijn meest opmerkelijke hobby. De in 2000 behaalde titel tijdens het Nederlands studentenkampioenschap vormde zijn theatrale hoogtepunt. Als promovendus vanuit de kinder-oncologie verrichtte hij van 2004 tot 2008 onderzoek op de afdeling tumor immunologie van het Nijmegen Centre for Molecular Life Sciences (afdelingshoofd prof. dr. Figdor). Onder begeleiding van prof. dr. Hoogerbrugge, prof. dr. Adema en dr. De Vries heeft hij translationeel onderzoek verricht naar immunotherapeutische behandelingsmogelijkheden van kinderen met kanker. Een belangrijk deel van het promotieonderzoek heeft hij afgerond in het *Ludwig Institute for Cancer Research* te Brussel onder supervisie van prof. dr. Coulie. Sinds 2008 is hij werkzaam als medisch immunoloog in opleiding in het UMC St. Radboud op de afdeling bloedtransfusie en transplantatie immunologie (opleiders prof. dr. Joosten en dr. Klasen). Daarnaast blijft hij als postdoc verbonden aan de afdeling tumor immunologie. Hij woont momenteel met vrouw en dochter in Nijmegen.

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J. Jacobs, H. van Bokhoven, F. van Leeuwen, C. Hulsbergen van de Kaa, I. de Vries, G. Adema, P. Hoogerbrugge and A. de Brouwer. MYCN regulation in neuroblastoma. Submitted.

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