Developing novel strategies for treating esophageal cancer: Dendritic cells immunotherapy, T cell responses and an exploration of the tumor microenvironment

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Chapter 1

General introduction

&

outline of the thesis

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INTRODUCTION

Esophageal carcinoma already was described at the beginning of the 19th century, and the first successful surgical resection was performed in 1913 by Frank Torek. Worldwide, esophageal cancer is the seventh leading cause of cancer death, squamous cell carcinoma being responsible for 95% of the cases. Esophageal cancer is relatively rare in Western countries such as Western Europe and in the United States. In the US esophageal cancer, ranks 19th among cancers in incidence, with 13,900 new cases per year, however, it is a particularly deadly malignancy, ranking 10th in cancer deaths, with 13,000 deaths per year. In the Netherlands, the incidence of Esophageal cancer increased from 6.4/100000 in 1994 to 8.9/100000 in 2003. Esophageal adenocarcinoma more commonly affects men than women, with lifetime risks of 0.75 and 0.26%, respectively. Until the 1970s, over 90% of esophageal cancers were squamous cell carcinoma, with adenocarcinoma of the esophagus being relatively rare. From then on the incidence of esophageal adenocarcinoma has been steadily increasing and, by the early 1990s, adenocarcinoma has become the most common cell type of esophageal cancer among whites. On the other hand, squamous cell cancers still predominates in blacks, and in most Eastern countries its incidence seems to be increasing. Currently in the Netherlands, esophageal adenocarcinoma accounts for more than 50% of all new cases of esophageal cancer. Its pathogenesis is linked to gastroesophageal reflux disease (GERD), and development of a metaplastic precursor lesion known as Barrett’s esophagus.

Risk factors for esophageal adenocarcinoma

While risks factors for squamous cell carcinoma of the esophagus have been identified, such as tobacco, alcohol, diet, poor socio-economic status, the risk factors associated with esophageal adenocarcinoma seems more complex. Barrett’s esophagus is regarded to be the precursor lesion and is associated with an increased risk for developing adenocarcinoma. Norman Barrett is credited with the discovery in 1950 of columnar epithelial cells lining the lower esophagus. Barrett originally believed that the columnar lining was the result of a short esophagus that drew the stomach tissue up into the thoracic cavity. Barrett’s esophagus is now recognized as a metaplastic condition in which squamous epithelial cells are replaced by intestinal-type of columnar cells as a consequence of gastro-esophageal reflux disease (GERD). Esophagitis, secondary to gastro-esophageal reflux disease, is the most common medical condition in Western countries with 30% of adults complaining of heartburn at least once per month,
a third of whom will have endoscopic evidence of esophagitis, of which 40% will improve spontaneously, while 50% have persistent esophagitis, and 10% develops metaplasia\textsuperscript{20}. It is supposed that reflux induces damage to squamous epithelial cells and causes (stem) cell proliferation, resulting in the replacement of squamous cells with columnar cells. This cell conversion may initially progress through the appearance of simple columnar epithelium before specialized intestinal cells are evident\textsuperscript{14,24}. Although different types of metaplasia may subsequently develop, at present Barrett’s esophagus is defined as an incompletely differentiated specialized columnar epithelium and is characterized by the presence of goblet cells. Barrett’s esophagus represents the first step leading to the development of EAC\textsuperscript{21,25-27}. In 1952, Morson and Belcher first described a patient with adenocarcinoma of the esophagus arising in columnar epithelium with goblet cells. In 1975, Naef et al. emphasized the malignant potential of Barrett’s esophagus\textsuperscript{28}. Metaplastic Barrett segments may vary in length, and are divided into short segments (less than 3 cm) or long segments (more than 3 cm). A long metaplastic segment represents the most important risk factor for progression to adenocarcinoma\textsuperscript{29}. The annual incidence of esophageal adenocarcinoma in patients with Barrett’s esophagus is estimated at 0.5% to 1\%\textsuperscript{30-32}. Histopathologic classification of dysplasia grade in Barrett’s esophagus [negative, indefinite, low-grade dysplasia (LGD), and high-grade dysplasia (HGD)] is the standard method of risk stratification\textsuperscript{33-35}. HGD has the highest morphologic association with progression to esophageal adenocarcinoma, and may be further subdivided into focal or diffuse types\textsuperscript{36,37}. A large number of genetic and epigenetic events contribute to the metaplasia-dysplasia-adenocarcinoma sequence, in the multistep process of malignant transformation\textsuperscript{38}. Most of the genes involved in the development of adenocarcinoma are normally responsible for cell cycle control. Alterations of the strictly controlled cell cycle may lead to dys-regulated proliferation, and, subsequently, to cancer\textsuperscript{39-44}.

**Clinical presentation and diagnosis of esophageal adenocarcinoma**

In a recent study, the most common presenting symptoms of EAC are dysphagia (74\%), weight loss (57\%) gastrointestinal reflux (20\%) odynophagia (16\%) and dyspnea (12\%)\textsuperscript{45,46}. Unfortunately, symptoms generally indicate relatively advanced disease. Early cancers are relatively asymptomatic and may be discovered as incidental findings at endoscopy. Dysphagia does not occur until the esophageal lumen is significantly compromised, and this therefore often points to the presence of a relatively advanced stage cancer. Generally dysphagia directs patients to endoscopy, which offers the opportunity for diagnosis, tissue sampling, and, when possible, therapy. Once an esophageal cancer is documented, staging is performed based on the TNM classification of the American Committee on cancer and the International Union Against Cancer. At first, it is advised to perform a computed tomography scan (CT) of
the abdomen, chest and pelvis to detect possible metastatic formations. If none is found, endoscopic ultrasonography (EUS) should be performed, to establish the status of the tumor (T and N status). It has been previously demonstrated that this procedure has the highest accuracy for T and N status classification\(^47\). The addition of fine needle aspiration of suspicious nodes increases the accuracy of N status to 93%. Positron Emission tomography (PET) may be useful for further staging of the EAC. In general, at the time of presentation up to 50% of patients have stage IV disease and are therefore not considered for surgical treatment.

**Treatment options**

The survival rate of patients with EAC is poor. Asymptomatic small tumors confined to the esophageal mucosa or submucosas are detected only by chance. Until recently, surgery was the only treatment of choice for these small tumors\(^48, 49\). Novel non invasive treatment options for removing mucosal cancers with a high success rate are endoscopic mucosal resection with or without ablative therapies\(^50-52\). Once symptoms such as dysphagia and weight loss are present, esophageal cancers have usually invaded the muscularis propria or beyond and may have metastasized to lymph nodes or other organs. Treatment strategies for EAC have been largely focused on surgery with curative intent\(^48, 53, 54\). Patients with localized disease are chosen to undergo surgery. Nevertheless, patients undergoing resection have a median survival of only about 15 to 18 months, corresponding to a five years survival of less than 20%,\(^55, 56\), therefore surgery with curative intent can be viewed as palliative in the majority of cases. To improve the outcomes of patients undergoing resection, the use of chemotherapy, radiation therapy, or both as adjuvant or neo-adjuvant treatments in conjunction with surgery have been explored. Survival highly depends on stage of disease, for instance, there is an overall 5 years survival rate of 85% for stage I, and of 65% for stage IIa disease\(^57, 58\), while stage IIb and III have 5 years survival rates of 51 and 14%, respectively\(^59\). Outcomes for more advanced stages are considerably worst and palliative strategies are the only consideration. Endoscopic treatment modalities such as stents or laser therapy play an important role in palliation of dysphagia, prevention of aspiration, and improvement in quality of life for many of these patients\(^60, 61\). After endoscopic palliation of adenocarcinoma at the distal esophagus or gastric cardia, mean survival has been around 140 days\(^62\). A recent study pointed out that palliative radiotherapy had similar or even slightly better results\(^63\). Improvement of surgical results over time has been attributed variably to change in epidemiology, patient selection, staging methods, surgical technique, and the use of additional treatments\(^53\).
Chapter 1

Combinations of surgery, radio- and chemotherapy
Based on existing trials, there was no clear evidence that preoperative radiotherapy improves the survival of patients with potentially resectable esophageal cancer\(^{64,65}\). It has been previously reported that definitive radiation therapy in combination with chemotherapy compared to radiation therapy alone resulted in an improvement in 5-year survival for the combined modality group (27% vs. 0%)\(^{66}\), while in a phase III trial comparing different dose of radiations, a higher radiation dose did not increase survival or local/regional control\(^{67}\). It has been previously demonstrated that chemotherapy using 5FU or cisplatin in combination with radiotherapy, followed by surgery compared to resection alone resulted in a modest patient survival improvement\(^{68-70}\). Many attempts have been done to explore the effect of combined therapy compared to surgery alone. The effects of preoperative chemotherapy have been evaluated in a randomized trial comparing this regimen to surgery alone. It was demonstrated that preoperative chemotherapy with a combination of cisplatin and fluorouracil did not improve overall survival among patients with epidermoid cancer or adenocarcinoma of the esophagus.\(^{71}\)

Despite several attempts to improve the outcome of EAC patients, no significant progress have been made and it is still a challenge to find novel and effective treatment to advance in the cure of EAC.\(^{69,72}\)

Immunotherapy: the beginning of a new era
In the past decades, a novel approach has been considered to be applicable to cancer therapy: immunotherapy using vaccines. The concept of immunotherapy is based on the body’s natural defence system, which protects us against a variety of diseases.

For many years, it was commonly believed that the immune system was effective only in combating infectious diseases caused by invading agents such as bacteria and viruses. More recently, evidence was found in favour of the hypothesis that the immune system may play a central role in protecting the body against cancer and in combating cancer that has already developed. The exact role is not well understood, but there is evidence that in many cancer patients the immune system slows down the growth and spread of tumors (Finn, O.J., Preventive role of existent immunity and developmental strategies for preventive vaccines, AACR 2007, Los Angeles, CA).

This concept came out for the first time in the 19\(^{th}\) century, when Dr. William Coley from the New York Memorial Hospital, showed that he could control the growth of some cancers. He cured several advanced cancers with injections of a mixed vaccine of streptococcal and staphylococcal bacteria known as Coley’s toxin\(^{73}\). The disillusion with conventional medicine, led him to wonder whether nature held a cure for cancer. He started to follow a case of a patient who presented recurrent sarcoma to the cheek. The extensive wound after surgery could not be closed and skin grafts were unsuccessful. Ironically, this failure to close the wound
would play a key part in the patient’s eventual cure. The wound became severely infected with erysipelas (*Streptococcus pyogenes*) and the patient developed a high fever. Little could be done to stop the infection, yet surprisingly, after each attack of fever the ulcer improved, the tumor shrank, and finally disappeared completely. Coley continued his studies based on the theory that nature can protect against cancer. Despite this concept, the tide began to turn against “Nature’s remedy” for cancer during the 20th century. Firstly, cancer surgery, like any other operation, became a sterile procedure after acceptance of Lister’s aseptic techniques in the late 1800s; secondly, by the time of Coley’s death in 1936, radiotherapy had become an established cancer treatment, and chemotherapy was rapidly gaining acceptance. These treatments could be more easily standardised than Coley’s approach.

Several decades later, the new era of tumor immunology began, when data were provided supporting the historical and recent evidence concerning the antagonisms between acute bacterial infections or their toxins and cancer and allied diseases. New data were provided for renewed incentives to undertake clinical programmes with mixed bacterial vaccines in many countries. A milestone in the concept of immunotherapy was introduced with the theory of Thomas and Burnett, in the midpoint of the twentieth century. They pioneered the famous concept of “immuno-surveillance of cancer”, to describe a mechanism that protects immune-competent hosts against tumors. Thomas and Burnett defined this as an “evolutionary necessity” and the existence of a mechanism to eliminate or inactivate dangerous mutated cells. (Burnet, F.M. Immunological factors in the process of carcinogenesis. *Br. Med. Bull.* 20, 154–158 (1964). With the availability of inbred strains of mice, the idea that tumors were immunologically distinguishable from normal cells could be critically tested. The demonstration that mice could be immunized against syngeneic transplants of tumor induced by chemical carcinogens, viruses or other means established the existence of “tumor specific antigens”.

Several research groups started to perform experiments, aimed by the wish to demonstrate the consistency and reliability of the concept, but this idea was largely abandoned when no differences in primary tumor development were found between athymic nude mice and syngeneic wild-type mice. Experimental tests failed to provide strong evidence for the mechanism of cancer immuno-surveillance, and because of this discordance, and most of the time disappointing results, the conclusion was drawn that tumors are not sufficiently distinct from normal tissue to activate the immune system. However, subsequent observations that nude mice do not completely lack functional T cells and that two components of the immune system, IFN-γ and perforin, help to prevent tumor formation in mice have led to renewed interest in a tumor-suppressor role for the immune response. In addition, new experimental approaches, such as gene targeting and transgenic mouse technology, and the possibility to produce highly specific monoclonal antibodies able to block immune components were introduced. Herewith, interest in the tumor
immuno-surveillance hypothesis is renewed, and the notion that the immune system regulates cancer development is experiencing a rebirth\(^79\). In the past few decades, the introduction of experimental ex-vivo mice models gave the opportunity to further provide data strongly supporting the idea that the host is supported by the immune-system in inhibiting tumor growth and development, although most recent data demonstrated that the immune-system is also responsible of promotion of tumor growth\(^80\). These new findings proposed a new dual role of the immune-system, and introduced a new concept of “cancer immuno-editing”\(^76,79,81\). This concept highlights the dual role of the immune system in promoting and inhibiting tumor development, and it was proposed for the first time from Ehrlich in 1909 who first proposed the idea that nascent transformed cells arise continuously in our bodies and that the immune system scans for and eradicates these transformed cells before they are manifested clinically (Ehrlich P. Ueber den jetzigen stand der karzinomforschung. *Ned Tijdschr Geneeskd* 1909; 5:73–290)\(^82\). Recently Dunn et al. hypothesized that this mechanism of cancer immuno-editing is based on three main events: elimination, equilibrium and escape (Fig.1, Taken from Dunn G.P. et al., Annu Rev Immunol. 2004;22:329-60). Immunosurveillance occurs during the elimination process, whereas the Darwinian selection of tumor variants occurs during the equilibrium process. In the first phase of elimination, once solid tumors reach a certain size, they begin to grow invasively through enhanced production of angiogenic factors. Invasive growth causes minor disruptions within the surrounding tissues that induce inflammatory signals leading to recruitment of cells of the innate immune system (NKT, NK, γδT cells, macrophages and dendritic cells) into the site. Chemokines, produced during the escalating inflammatory process, recruit more NK cells and macrophages to the site. The process of elimination proceeds though developments of adaptive immune responses, i.e. CD8+ T cell responses\(^76\).

![Diagram of immune response phases](image)

**Fig. 1**
The next step in cancer immunoediting proceeds to the equilibrium phase in which a continuous sculpting of tumor cells produces cells resistant to immune effector cells. This process leads to the immune selection of tumor cells with reduced immunogenicity. These cells are more capable of surviving in an immunocompetent host, which explains the apparent paradox of tumor formation in immunologically intact individuals. On the other hand, some mice models of cancer have shown that the inflammatory actions of the immune system can promote tumor development and/or growth, suggesting a strict correlation between inflammation and tumor progression. Furthermore, tumors evolve mechanisms to escape immune control by a process called immune editing, which provides a selective pressure in the tumor microenvironment that can lead to malignant progression. This results in clinically observable malignant disease that, if left unchecked, results in the death of the host.

Dendritic Cells: a potent tool for cancer immunotherapy

The Dendritic Cell (DC) system of antigen-presenting cells (APCs), is the initiator and modulator of the immune response. First visualized as Langerhans cells (LCs) in the skin in 1868, the characterization of DCs began only 25 years ago. It was known that ‘accessory’ cells were necessary to generate a primary antibody response in culture, but it was only once DCs were identified and purified from contaminating lymphocytes and macrophages that their distinct function as APCs became apparent. DCs are unique APCs, because they are the only ones that are able to induce primary immune responses, thus permitting establishment of immunological memory. DC progenitors in the bone marrow give rise to circulating precursors that home to tissues, where they reside as immature cells with high phagocytic capacity. Following tissue damage, immature DCs capture antigens (Ags) and subsequently migrate to the lymphoid organs, where they select rare Ag-specific T cells, thereby initiating immune responses. DCs present Ags to CD4+ T-helper cells, which in turn regulate the immune effectors, including Ag-specific CD8+ cytotoxic T cells and B cells, as well as non-Ag-specific macrophages, eosinophils, and Natural Killer cells (Fig.2). Moreover, DCs educate effector cells to home to the site of tissue injury.

DCs are typically defined on a combination of parameters that include morphology, phenotype, cytokine secretion pattern, immunostimulatory capacity, chemokine and chemokine receptor pattern, and migration in response to chemotactic stimuli. The DC phenotype varies, depending on stage of maturation and differentiation. For example, CD1a is preferentially expressed on human myeloid DCs, whereas CD83 is typically up-regulated in response to activation stimuli such as tumor necrosis factor alpha, Toll like receptor ligands, Cytidylyl-2p,5p-phosphoryl guanosine (CpG), double stranded RNA, prostaglandin E2, or T cell derived signals including CD40 ligand and IFN-γ. DCs express as well co-stimulatory molecules
such as CD80 (B7.1) and CD86 (B7.2), dectin and CD40. DCs are characterized by potent immune-stimulatory capacity, which can be detected in a mixed leucocyte reaction (MLR), and by the ability to prime antigen-specific lymphocytes, both in vitro and in vivo. These capacities are enhanced upon exposure to activating stimuli. DC immunogenicity is largely determined also by their capacity to secrete cytokines such as TNF alpha, IL-6, IL-12, IL-15 and IL-8, which contribute to activate T cells and prime the subsequent immune-response (Fig.2). Different subsets of DCs can also control the type of immune response, by stimulating
helper or cytotoxic T-cells that in turn will produce different types of cytokines, for instance: IFN-γ production by T-helper 1 cells, IL-4 by T-helper 2, or IL-10 by regulatory T-cells. This means that DCs have a complex constitution that under different micro environmental conditions can induce divergent immune responses, on one hand they can induce immunity, and on the other hand they may mediate tolerance. At least three distinct types of DCs exist: myeloid DCs, lymphoid DCs and Langerhans cells (LCs). Evidence for the myeloid origin of DCs comes mainly from in vitro studies in which myeloid-committed precursors give rise to both granulocytes/monocytes and myeloid DCs under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF). Transfer of a population of purified lymphoid precursors into irradiated hosts resulted in the development of T cells, B cells, NK cells, and DCs that express CD8α, but not cells of the myeloid lineage. Three subsets of DC precursors circulate in the blood: CD14+ monocytes, lineage negative CD11c+ precursor DCs, and CD11c+ precursor DCs. Monocytes can differentiate into cells displaying features of immature DCs or macrophages in response to GM-CSF and IL-4 or M-CSF, respectively. The CD11c+ subsets contain precursors of interstitial DCs, LCs and macrophages. Distinct factors regulate the survival and differentiation of CD11c+IL-3Rα+ DC precursors, originally described as plasmacytoid T cells or plasmacytoid monocytes. These cells die rapidly after isolation and are critically dependent on IL-3 for survival and CD40L for maturation.

The role of T helper cells
DCs are highly capable of inducing T-cell responses, and the interaction of these two cell types, is determinant for the type of immune response that will be raised. The capacity of the DCs to stimulate T-cells depends on many factors, such as surface expression of co-stimulatory molecules, and the cytokine microenvironment. It is curious to note that T cells may play an important role in activating DCs, thus further enhancing the T cell stimulatory capacity of the DCs. It has been demonstrated indeed that CD40 ligand, expressed by T-cells, increase DC viability and induce DC maturation. DCs can produce IL-12, a key cytokine for the generation of Th1 responses. On the other hand, DC function can be suppressed by the production of IL-10, which induces apoptosis, reduced capacity to stimulate T-cells and decreased production of IL-12. While development of IFN-γ and IL-2 producing Th1 cells leads to protective anti-tumor immune responses, Th2 cells producing IL-4 and IL-10 may be associated with non-protective responses. Furthermore, as Th1 and Th2 cells have been considered as representing the two extremes of T-cell polarization, a third CD4+ T-cell population has been recently singled out. These T-cells, named the T regulatory cells (Tr1) has been demonstrated to produce high levels of the cytokine interleukin-17 (IL-17), which has a major role in various models of
immune-mediated tissue injury. With this observation, a new perspective was introduced in the world of immunology. Analysis of T-cell clones isolated from the mouse CD4+ T-cell populations that were repeatedly stimulated with OVA peptide and splenic APCs in the presence of IL-10 showed that half of the CD4+ T-cell clones displayed this cytokine profile. They produced high levels of IL-10 and undetectable levels of IL-2 and IL-4. The observation that Tr1 cells secrete high levels of the immunosuppressive cytokine IL-10, and low levels of the T-cell growth-promoting cytokines IL-2 and IL-4, suggest that antigen-specific activation of Tr1 cells may result in inhibition of antigen-specific proliferation of other T cells. Indeed, co-culture experiments using a transwell system confirmed this notion for both human and mouse T cells. The recent identification of these Tr1 cells actually develops numerous observations of ‘suppressor’ CD4+ T-cells that may also play an important role in tumor ‘anergy’. The inhibitory factor appears to be IL-10, a cytokine capable of converting DC/APC function to the induction of antigen-specific anergy, thus leading to the state of tolerance against tumor tissue.

The choice of antigen source
Most, if not all, tumors are antigenic and a majority of identified tumor associated antigens (TAA) are non-mutated, overexpressed or inappropriately expressed, tissue differentiation antigens. Another category of TAAs represented in several tumors are re-expressed embryonic antigens and growth factors, such as MAGE, gp-100, and HER-2, which present an aberrant expression, or overexpression, mostly via gene amplification. The most notable examples are in melanoma in which major proteins implicated in the tumor-specific immune response are non-mutated re-expressed embryonic antigens, e.g., MAGE and gp100. Tumor antigens can be as well classified according to the type of immune response they elicit: humoral, cellular, CD4+ (T helper), or CD8+ cytotoxic T lymphocyte (CTL) responses. As will be discussed below, the fact that a tumor antigen elicits a tumor-specific immune response does not necessarily mean that the immune response will cause the rejection of the tumor in vivo. Thus, from a vaccination perspective, the question is which tumor antigen can or is better at inducing a clinically beneficial response. Such antigens are referred as “tumor rejection antigens”. Tumor antigens can be poor, intermediate, or strong tumor rejection antigens, describing quantitatively the effect of the immune response on tumor growth. Several different strategies have been extensively explored to deliver antigens into ex-vivo manipulated of DCs to enhance their potency to stimulate T cells. DCs can be loaded with synthetic peptide epitopes derived from known TAAs such as MUC1, HER-2/neu, tyrosinase, telomerase, CEA, p53, MAGE, or Melan-A/MART. Major drawbacks of using peptides are the restriction to some HLA class I alleles, the need to
determine the expression of the target antigen by a particular tumor, the limited number of defined TAA and the possibility that targeting single or few tumor epitopes may impede the detection of tumor cells that may have downregulated expression of these antigens. Moreover, additional biologically important epitopes may be missed, as binding affinity for a particular HLA allele does not translate directly into immunogenicity. Using MHC class I-restricted peptides also ignores the role of MHC class II-restricted T helper cells in initiating and sustaining an immune response. Another strategy which can be applied to deliver tumor antigens to DCs is based on gene transfer methods that result in antigen processing in the MHC class I pathway of DC and presentation to CD8+ T cells. DCs can be transduced with DNA, with or without liposomal encapsulation, which has been tried with varying success. RNA derived from tumors or encoding tumor antigens can also be transfected into DCs leading to the generation of primarily class I MHC restricted responses. When these approaches are used, the vaccine may contain multiple antigens, increasing the probability of inducing immunity to more than one tumor-associated antigen. Although the target proteins are initially undefined, with this approach, the immunogen can be identified afterwards. Theoretically, with such an approach there is increased potential for the induction of a destructive autoimmune response to antigens expressed on normal tissues. In practice, there is a 'self defence' mechanism against the auto immune responses through selective destruction of T-cell that recognize auto-antigens in the thymus during fetal development. Furthermore induction of autoimmune response by such antigens presumably would be limited to tumor cells bearing epitopes of the self antigens and TAAs, limiting the risk of collateral damage to normal tissues. If such proteins arise in the tumor after thymic development, antigen-reactive T cells could exist in the repertoire as they may have avoided thymic deletion. The mechanisms involved in these observations are poorly understood and future studies will enlighten us on this matter. One important limitation of most studies is that not all ex-vivo observations of immune responses can be extrapolated to the human situation. While animal models have been used frequently to examine the immunogenicity of tumor-associated antigens, there are as well significant caveats in extrapolating animal data to humans. For instance, many of the animal tumor antigen models use human proteins that are foreign to the animal (e.g. carcinoembryonic antigen, or HER-2/ neu in mice), which by definition will induce a strong immune response in the animal. Transgenic tumor models could be used to address this issue although differences in T cell receptor repertoire remain an important confounding factor.
DC dose, frequency and route of injections.

In human trials published so far, several different dosages at different intervals were investigated without giving outstanding differences in results. It has previously been demonstrated that in vitro-generated mature, in contrast to immature DCs, efficiently migrate into the T-cell areas of lymph nodes of melanoma patients and this difference was confirmed by in vitro studies. These findings demonstrate that the ability of DCs to induce an efficient immune response correlates with their ability to migrate both in vitro and in vivo, and this phenomenon is correlated with molecular changes, for instance up-regulation of antigen-presenting MHC molecules and co-stimulatory molecules, a switch in chemokine receptor expression with down-regulation of receptors for inflammatory chemokines, and up-regulation of receptors for chemokines produced in secondary lymphoid organs. Another important parameter to consider when preparing a vaccination strategy is the ability of the DCs to migrate from the site of injection to the lymph nodes to be able to encounter resting T cells and prime immune responses. In 2003 de Vries et al. demonstrated that regardless whether DCs are injected intranodal or intradermal, mature DCs are migratory both in vitro and in vivo. Despite clear evidence of the capacity of the injected DCs to migrate to lymph nodes, the optimal route for administering DC vaccines is still a subject of debate. Historically, melanomas were the first cancers treated by DC therapy and traditionally intradermal vaccination is the most frequently applied route of vaccination. Several animal and human studies however show effective anticancer response in case of intratumoral injections. In a randomized phase I trial patients with metastatic melanoma received peptide pulsed DCs either intravenously, intranodally or intradermally. The intranodal route induced significantly higher rates for the novo development of CD8+ T cells as determined by MHC Tetramer staining compared with the other routes. DC therapy for treating malignancies within the gastro-intestinal tract including esophageal cancers has a relatively short history. These cancers have the unique feature that they arise from the mucosa. The esophagus is furthermore provided with an extensive network of lymphatics. This is reflected in aggressiveness of these cancers characterized by early lymph node metastasis of even small mucosal cancers. It is very likely that intramucosal vaccination for treating esophageal cancer may be as effective or perhaps superior to intranodal injections. Despite the frequently raised criticism that intranodal injection might greatly disturb the node architecture, migration to subsequent nodes has been observed and follows the physiological path through lymph vessels. For esophageal cancer the best route for administering the vaccines is yet not known, therefore, intranodal versus intramucosal administration of peptide-loaded DC vaccines remains to be explored.
Results from clinical trials employing Dendritic Cell immunotherapy

The first attempt to use DCs vaccines was done by Hsu et al to treat B-cell lymphoma\(^{141}\). After this pioneer study, many others attempted to develop strategies to apply and improve DCs immunotherapy for cancer patients, particularly for melanoma and prostate cancer\(^{142-147}\). Although the general experience suggest now that DCs can be administered safely, still clinical responses, either complete remission, partial remission, or stable disease, can be observed in only few patients\(^{148-151}\). O’Rourke et al. observed complete remission in three patients after vaccination in melanoma patients using DCs loaded with irradiated autologous tumor cells\(^{149}\). Holtl and colleagues showed that loading mature DCs with tumor lysates, 2 out of 35 renal cell carcinoma patients could respond completely, one partially, and 7 could reach stable disease\(^{148}\). Schuler-Thurner and colleagues included 16 melanoma patient in a study employing mature DCs loaded with melanoma peptides, and could observe one complete remission, and 8 partially recovered patients\(^{142}\). Although these studies are hardly comparable because of the differences in criteria of inclusions, type of antigen source, route of vaccine delivery, the results are indicating that DCs are safe, immunogenic, and induces T-cell responses and remission in few patients. Many more studies have to be performed to improve such an appealing strategy for curing cancer, especially to understand what determines such unsatisfactory results in terms of clinical responses.

The Tumor Microenvironment

Despite major advances in the understanding the components of the immune system, it has been broadly demonstrated that the poor clinical outcomes and low efficacy of immunotherapy against cancer and the failure in curing cancer is due to an immuno-suppressive network, created as a consequence of pathological interactions between cancer cells and host immune cells\(^{80,152}\). Tumor resistance is a result of an alteration in the production of specific factors, for instance co-stimulatory and co-inhibitory molecules, an imbalanced activation of effector T-cells and regulatory T-cells (Fig.3, taken from W.Zou, Nat Rev Cancer. 2005 Apr;5(4):263-74). This balance is especially altered in patient with advanced stage cancer, which presents high levels of inflammatory molecules, cytokine, chemokines, and tumor infiltrating T-cells\(^{80}\). There is rising evidence that the tumor microenvironment certainly inhibits the development and function of DCs, by over-expressing immuno-suppressive molecules such as COX-2, TGF-beta, IL-6, M-CSF, IL-10, PGE-2, and VEGF\(^{153,154,155}\); on the other hand, there is a lack of immuno-stimulatory factors, such as GM-CSF, IFN-$\gamma$ and IL-4. This environment enables cancer cells to escape from effector T-cells responsible of recognition and attack of the tumor mass. Furthermore, chemokines are secreted into the tumor microenvironment by tumor-infiltrating inflammatory cells as
well as by the tumor cells themselves. Evidence from murine models and human tumors suggests that CC chemokines are major determinants of macrophage and lymphocyte infiltration. In melanoma, carcinoma of the ovary, breast, and cervix, and in sarcomas and gliomas, receptors for chemokines (CCR and CXCR) are expressed both by infiltrating leucocytes and by cancer cells. The leucocytes may
lose receptor expression once they are exposed to inflammatory cytokines in the tumor microenvironment, as shown for CCR2 on tumor associated macrophages in ovarian cancer. Recently, it has been demonstrated that regulatory T-cell mediated immuno-suppression is one of the crucial tumor immune-evasion mechanisms and the main obstacle of successful tumor immunotherapy. It has been demonstrated that depleting T-regulatory cells (T-regs) in human cancer, including ovarian cancer, in combination with supplied therapies, improves immunity and may be therapeutic. CD25 is constitutively expressed in effector T-cells and T-regs. Early studies demonstrated that in vivo administration of a CD25-specific antibody suppressed growth of several progressive tumors. Elimination of CD25-expressing T cells, which constitute 5-10% of peripheral CD4+ T cells in normal naive mice, elicited potent immune responses to syngeneic tumors in vivo. Tumors employ several strategies to evade immune-response, including tumor-induced impairment of antigen presentation, the activation of negative co-stimulatory signals, and the elaboration of immunosuppressive factors. Moreover, recent years have witnessed increasing interest in immunosuppressive cells of myeloid origin, especially about their role in cancer immune-escape. Immunosuppressive myeloid cells accumulate in large numbers in tumor-bearing mice, in several experimental models, as well as in patients with breast, lung, prostate, kidney, head and neck, and other types of cancer, they are produced in response to a variety of tumor-derived cytokines and are a heterogeneous mixture of myeloid cells at different stages of differentiation. For this reason, it is of basic importance to find new strategies to move the balance of all these factors in favor of the tumor immunity, either through boosting the activation of specific potent cytotoxic T-cells, exploring novel strategies for enhancing DC therapy, or providing effector cytokines such as IL-2, IFN-alpha, IL-12, or by blocking the suppressive environment via inhibition of immunosuppressive cells, such as Tregs, myeloid derived suppressor cells, plasmacytoid DCs, immature DCs, and inhibition of immunosuppressive molecules, such as COX-2, IL-6, PGE2, TGF-beta, EGF, Cyclin D1 and many more.

**Combining DC therapy with anticancer drugs targeting several known substrates**

It is anticipated that the efficacy of DC therapy will greatly benefit through combination with anti-cancer drugs that either modulate the tumor environment or inhibit growth factors. Therefore, the efficacy of several strategies combining DC vaccines and several applicable and ready available anticancer drugs against several pharmacological substrates known to be highly expressed in esophageal adenocarcinomas, such as Her-2/neu, COX-2, VEGF and TGF-beta, are currently being tested. Several anticancer drugs can potentially be interesting to be tested in combination with DC immunotherapy induced CTL responses.
It has been previously shown that Celecoxib, a specific inhibitor of COX-2, reduces cell viability and induces apoptosis in vitro and, furthermore, evaluation in vivo has shown that Celecoxib administered as neoadjuvant treatment in patients with esophageal adenocarcinoma induced a decrease in COX-2 expression, without particular adverse effects. Another important mechanism of tumor progression is angiogenesis, mediated by several factors, in particular by vascular endothelial growth factor (VEGF), which is most essential pro-angiogenic growth factors expressed by most cancer-cell types and certain tumor stromal cells. It has been demonstrated that blocking this mechanism via specific antibodies against VEGF, such as Bevacizumab, significantly improve clinical benefit for several solid tumor patients. The first report of a large, prospective randomized trial of anti-VEGF therapy in patients with metastatic breast cancer (MBC), which demonstrated the benefit of adding the monoclonal antibody Bevacizumab to the chemotherapeutic agent paclitaxel was performed in 2005. The success of this trial provided proof of principle that inhibition of angiogenesis has the potential to enhance the effectiveness of treatment of breast cancers, and perhaps also for other solid tumors bearing VEGF overexpression. Therefore, it is of interest to evaluated the use of bevacizumab in esophageal cancer, which has high expression of this factor.

**Immunotherapy in combination with blocking of the HER-2 oncoprotein.**

It is now generally accepted that although many advances have been made in the field of cancer vaccination using DCs, the present clinical outcomes of patient trials anticipate that DC therapy alone for treating advanced stage disease will be below expectation. Therefore, the possibility to combine the efficacy of DC therapy with other treatment strategies to improve clinical responses is attractive. Several approaches have been explored, such as the combination of chemotherapy and immunotherapy, or strategies to increase immunity for instance through combining immunotherapy with depletion of CD25+CD4+FOXP3+ T cells. A potent immunomodulatory drug that might be eligible to be combined with DC immunotherapy is Herceptin (Trastuzumab). Trastuzumab is a fully humanized antibody designed to block the function of the HER-2 oncoprotein specifically targeting the HER-2 extracellular domain. Trastuzumab, as such, induces growth inhibition against HER-2–overexpressing tumors. C-erbB2, the (onco) gene that codes for HER-2, can be over-activated by gene amplification with an increased expression of the normal gene product. As a protein which is normally found in cells and is overexpressed in tumor, the HER-2/neu protein represents a typical example of a rather new concept in tumor immunology, which suggests that self-proteins can serve as tumor antigens. Thus, a current issue for the development of cancer vaccines is how best to induce T-cell immunity to “self” tumor antigens. It has been reported in several studies that HER-2/neu is overexpressed in 30 to 80% of esophageal adenocarcinomas, and in
many other gastrointestinal cancers\textsuperscript{176,177}. Lately, there has been much attention to HER-2/neu expression in human breast cancer. It is known that HER-2/neu overexpression can be found in 30-40\% of this common malignancy\textsuperscript{178,179}. In several studies it has been demonstrated that selected HER-2 peptides are immunogenic as they are processed as TAAs and can induce specific CTL responses against cancer cells in vitro and in vivo\textsuperscript{171,178,180-184}. These reports support the hypothesis that HER-2 immuno targeting might form an attractive strategy to treat HER-2 overexpressing tumors. The humanized monoclonal antibody Trastuzumab exhibits potent growth inhibitory activity against HER-2/neu overexpressing tumors\textsuperscript{185}. It has been demonstrated that Trastuzumab has survival effects on HER-2/neu overexpressing breast cancer\textsuperscript{186,187}. The use of Trastuzumab in combination with for instance chemotherapeutic agents has been reported to significantly improve treatment efficacy\textsuperscript{188}. Although many studies demonstrated the efficacy of Trastuzumab as treatment for improving clinical outcomes, the mechanisms through which this antibody works is still unclear\textsuperscript{189}. Amongst the mechanisms that have been proposed are: the reduction of PI3K pathway activation, thus promoting arrest of proliferation and apoptosis\textsuperscript{190}; the inhibition of angiogenesis\textsuperscript{191}; inhibiton of HER-2 cleavage\textsuperscript{192} and antibody-dependent cellular cytotoxicity (ADCC)\textsuperscript{185,193,194}. Several questions have to be answered regarding the mechanism of this antibody, however, there is sufficient evidence that support the importance of developing combinatorial strategies including HER-2/neu blockade for improving cancer patient outcomes.

**Aim and structure of this thesis**

The aim of this thesis is to investigate novel and more advantageous strategies to treat esophageal cancer, particularly focusing on Dendritic Cell (DC) immunotherapy. DC immunotherapy is an attractive strategy which could represent an alternative treatment approach for such an aggressive cancer. DC immunotherapy is still under development and in numerous aspects needs to be further optimized in preclinical studies. This thesis aimed at resolving a number of important difficulties in this field, in order to prepare for a phase I/II clinical trial. In order to achieve this purpose, we explored different strategies.

**Chapter 2** aims on improving the efficiency manufacturing DCs from peripheral blood monocytes. This chapter describes a methodology to electroporate freshly isolated monocytes to obtain mature DCs, which in turns will be capable of presenting antigens to T lymphocytes and induce an immune response to those antigens. We demonstrated that this method is effective in obtaining immune-potent antigen presenting DCs with a proper immuno-phenotype, and with a proper immuno-stimulatory capacity and migration ability.

**Chapter 3** aims on developing a better human model to monitor for DC mediated CTL responses. This chapter reports the establishment of a unique ex-
vivo autologous readout method, in which primary cell cultures from esophageal cancer and normal tissues of patients are established. Subsequently using the strategy described in chapter 2, autologous DCs and CTLs are obtained and immune-response, and potential adverse effects, are monitored in the autologous cultures of the esophageal cancer patients.

Chapter 4 reports a characterization of the immunological and molecular esophageal tumor microenvironment, which is of basic importance to monitor when applying immunotherapy to cancer patients. In addition, these factors represent also an explanation of why cancer vaccines at this point do not have convincing and successful results.

Chapter 5 aims on evaluating DC immunotherapy and Trastuzumab as a combinatorial strategy for treating esophageal adenocarcinoma patients. This chapter reports our findings about the action of Trastuzumab, the humanized monoclonal antibody against HER-2, in EAC cell lines which overexpress HER-2, and the prospective to use this antibody as a combinatorial treatment in EAC patients.

Chapter 6 describes the potential role of BMP-4, a factor previously found by our group to be up-regulated in Barrett biopsies, in the mechanism of development of intestinal non-specialized columnar type of cells, which in turn can develop to a specified columnar type of epithelium typical of Barrett’s Esophagus.
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Chapter 2

An improved protocol for generation of immuno-potent Dendritic Cells through direct electroporation of CD14+ monocytes

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ABSTRACT

In this study we demonstrate a novel protocol showing that electroporation of CD14+ monocytes directly isolated from blood with Green Fluorescent Protein (GFP) RNA results in a 3-fold higher yield of antigen presenting Dendritic Cells (DCs) when compared to conventional methods employing immature DCs for electroporation. We further show a stable electroporation efficacy resulting in 60% of GFP positive cells. Expression of co-stimulatory molecules and maturation markers such as CD80, CD86, CD83 as well of the chemokine receptor 7 (CCR7) was found in 90% of the mature DCs. Importantly, production of IL12p70 was 10 times higher in cells electroporated at the monocyte stage compared to cells electroporated at the immature DC stage. Stimulation of autologous naïve lymphocytes by DCs electroporated at monocytes stage elicited proliferation of CD8+ T-cell with 7 fold increase in IFN-γ release. Blocking of the MHC-Class I molecules significantly inhibited the IFN-γ release, indicating that antigen presentation was MHC-Class I mediated. In summary, electroporation of CD14+ monocytes with RNA results in a high yield of antigen presenting DCs with high immuno-stimulatory capacity and antigen presentation on MHC class I molecules. This improved method may represent an attractive approach for RNA based DC immunotherapy.
INTRODUCTION

Cancer immunotherapy is a novel approach which has been investigated in the last few decades as an alternative therapy against oncogenic disease. Dendritic Cells (DCs) are professional antigen presenting cells (APCs) with a unique ability to capture and process tumor associated antigens for inducing T-cell mediated immune responses. In the last few years, the possibility to generate functional DCs from human peripheral blood monocytes that can be loaded with tumor antigens using various protocols has been explored extensively.\(^1\) In the past, most antigen-based vaccines employing either known tumor antigens or whole-cell tumor lysates to initiate a specific T-cell response exhibited limited immunogenicity, raising concern that human cancer cells might possess inherently poor antigenicity, or that antigen-presentation and T-cell activation may be suppressed in cancer patients.\(^6\) Moreover, there is raising concern that the tumor itself is surrounded by an unfavorable environment that can induce immunotolerance and allow the tumor to escape from immune surveillance inducing deleterious effect on T-cells.\(^7,8\) For these reasons, several approaches have been employed to obtain high yields of immuno-potent antigen presenting DCs. In the development of DCs vaccines, one of the first concerns is to obtain sufficient amounts of DCs that fully mature with preserved immuno-stimulatory capacity.\(^9\) Secondly, the DCs have to be loaded with tumor associated antigens able to induce a specific T-cell response against cancer cells. DCs have been successfully loaded with antigens using different methodology, such as lipofection, passive pulse, or transfected through electroporation with DNA, total RNA or mRNA of defined tumor antigens.\(^10-12\) In general most assays employ blood or bone marrow monocytes that are first stimulated to become immature or mature DCs before cells are loaded with the antigens.\(^13\) Electroporation proves to be a more efficient and frequently applied method for antigen loading of DCs compared to other methods.\(^10\) This procedure, however, is associated with a significant loss of cells. It appears that in vivo the capacity to interiorize and process antigens is a constitutive property of immature DCs when present in non-lymphoid organs.\(^14\) Therefore most researchers rely on employing immature DCs for electroporation. Yet, electroporation of monocytes directly isolated from blood has been described as well.\(^15\) Electroporating monocytes directly isolated from peripheral blood without any pre-incubation, would give the possibility to avoid several laborious cell culturing and harvesting steps leading to a more efficient protocol for obtaining antigen loaded DCs. Moreover, monocytes may be more resistant against the electroporation procedure itself that may lead to a significant higher recovery of cells after the electroporation technique. It is, however, not known whether electroporated monocytes would efficiently present the electroporated antigens and mature into potent APCs.
this study we present a novel efficient protocol for obtaining high yields of potent DCs through electroporation of monocytes directly isolated from peripheral blood. We first examined whether we could achieve stable electroporation of monocytes with Green Fluorescent Protein (GFP) RNA and maintain a persistent expression of the antigens during the maturation process into DCs. We measured the cell recovery rate of monocytes after the electroporation procedure and compared it to that of immature DCs. We tested whether the electroporated monocytes subsequently matured into effective immuno-potent DCs, expressing co-stimulatory molecules and exhibiting a mature phenotype. We looked into the production of pro- and anti-inflammatory cytokines such as IL-12p70 and of IL-10 and compared our protocol with the more conventional method, in which immature DCs are employed for electroporation. Finally, we studied the immuno-stimulatory ability of the mature DCs for inducing a CD8+ T-cell response, measuring the CD4+/CD8+ T-cell ratio, T-cell viability and T-cell proliferation and looked into IFN-γ production. This study demonstrates that electroporation of monocytes directly isolated from blood is an efficient procedure that results in a 3 times higher yield of immuno-potent DCs when compared to the more conventional electroporation of immature DCs. The presented protocol may considerably facilitate the manufacturing of DCs and may greatly enhance further development of future DCs RNA-based vaccines.

MATERIALS AND METHODS

Isolation of monocytes and lymphocytes from peripheral blood
Human monocytes were isolated from peripheral blood of healthy volunteers using the Ficoll-Percoll gradient separation method. Sixty four ml of blood was drawn from each volunteer. A first separation of peripheral blood mononuclear cells (PBMCs) was done using Ficoll-Hypaque solution (Amersham, Pharmacia, Piscataway, NJ, USA). A further separation of monocytes from lymphocytes was done using a Percoll (Amersham Biosciences Europe Freiburg, Germany) gradient separation, following manufacturer’s protocol: briefly, PBMCs were washed in RPMI 1640 medium (BioWhittaker-Cambrex Bioscience, Walkersville, MD, USA) at 1500 rpm for 5 minutes twice. At the mean time, Percoll was mixed with 10X Phosphate Buffer Saline (PBS) to obtain Standard Isotone Percoll solution (SIP), and then with IMDM medium (BioWhittaker), to obtain three solutions at different concentration (60, 47.5 and 34% SIP). PBMCs were then re-suspended in 2.5 ml 60% SIP, then 5 ml 47.5% SIP and finally 2 ml 34% SIP were added to the 60%SIP cell suspension and a centrifugation at 3100 rpm for 45 minutes was performed. Once monocytes were separated from lymphocytes, both populations were re-suspended in IMDM and washed twice. Monocytes
and lymphocytes were counted and either used immediately or cryo-preserved in a DMSO/FCS (2:8) solution (Merck, Darmstadt, Germany; GIBCO BRL, Grand Island, NY, USA, respectively).

**Electroporation of monocytes with In Vitro Transcribed (IVT) GFP-RNA**

Monocytes characterized as CD14+, CD45+, CD80-, CD86-, CD83- and CD209- (Fig. 2A) were used for electroporation with IVT GFP-RNA as follows: Monocytes freshly isolated from blood were washed twice in IMDM and electroporated using the Amazax cell line Nucleofector Kit V (Amazax GmBh, Cologne, Germany): 2x10⁶ cells were mixed with Nucleofector transfection solution V, and 5 μg/ml of IVT GFP-RNA or 1 μg/ml of autologous normal RNA (control); the program U16 of the Amazax transfection machine was used. Likewise, immature DCs, harvested after six days of culturing and characterized as CD14- and CD80+, CD83+, CD86+ and CD209+, were electroporated with IVT GFP-RNA using the same procedure as described above.

**Maturation of electroporated monocytes into DCs.**

For maturing the monocytes into DCs the protocol as described by Braat et al.¹⁶ was used. Upon electroporation monocytes characterized as CD14+, CD45+, CD80-, CD86-, CD83- and CD209- (Fig. 2A) were immediately cultured in IMDM medium with 10% FCS and 2% antibiotics (GIBCO) in 24 wells plate in a concentration of 5x10⁵ cells/ml and left for 1 hour at 37˚C and 5%CO₂ for the adherence step. After one hour from the electroporation, cells were washed with IMDM, than 1000 U/ml of IL-4 and 800 U/ml GM-CSF were added to initiate the maturation process. After six days at the stage of immature DCs, the pro-inflammation cytokines IL-1β and TNF-α and LPS (10 ng/ml, 25 ng/ml, 0.02 ng/ml respectively) were added to further enhance the maturation process. After 2 more days of maturation, cells proved to have a mature phenotype characterized as: CD14-, CD45-, CD80+, CD86+, CD83+ and CD209+ (Fig 2B).

**Detection of markers in monocytes and DCs**

Detection of CD14, CD45, CD80, CD83, CD86, CD209 and CCR7 in monocytes, immature and mature DCs was as follows: cells were harvested, washed and resuspended in a concentration of 1x10⁶ in FACS buffer (5 g BSA, 0.1 g NaN₃, 100 mM EDTA in 1 L PBS), then incubated with various fluorochrome-conjugated antibodies on ice for 30 minutes in the dark, then washed again and analyzed with the FACSCALIBUR apparatus (Becton-Dickinson, Franklin Lakes, NJ, USA); antibodies PE conjugated, specific for CD14, CD45, CD86, CD80, CD83, CD209 and CCR7 (BD, San Jose, CA, USA) were used, in a concentration of 1:25.
Electroporation efficacy in monocytes as measured by GFP expression during maturation into DCs

The electroporation efficacy of GFP-RNA was assessed through measuring of GFP expression by FACS analysis. Briefly, the procedure was as follows: cells were harvested and spun down at 1500 rpm for 10 minutes, then re-suspended in FACS buffer in a concentration of $1 \times 10^6$ cells/ml and sorted by the FACS apparatus for direct measurement of GFP expression. GFP expression was measured in monocytes before electroporation (day one) and daily on eight subsequent days after electroporation, during the maturation of the monocytes into immature and mature DCs. For each time point GFP expression was measured in three different samples.

Cell loss and recovery due to culturing and electroporation of monocytes and immature DCs

For assessing cell loss due to the process of culturing and electroporation of cells the Trypan Blue viability test was used following the manufacture’s procedure. Two millions monocytes freshly isolated from blood were directly electroporated and immediately plated in 24 well plates in a concentration of $5 \times 10^5$ cells/well, at 37°C and 5%CO$_2$. After 8 days of culturing, at the mature DC stage, cells were harvested and counted to assess the recovery rate after electroporation. To obtain DCs at the immature stage, monocytes freshly isolated were directly plated in 24 well plates at the concentration of $5 \times 10^5$ cell/well, and stimulated for six days to differentiate into immature DCs (see above). After six days of culturing, at the immature DC stage, cells were electroporated and immediately plated in 24 well plates in a concentration of $5 \times 10^5$ cell/well, at 37°C and 5%CO$_2$. After 48 hours, cells were harvested and counted to assess the recovery rate of immature DCs after electroporation.

Stimulations of autologous lymphocytes with mature DCs.

For stimulation of the autologous lymphocytes, lymphocytes and DCs were incubated in a proportion of 1:4 ($5 \times 10^5$ DCs and $2 \times 10^6$ lymphocytes) in 24 wells plate in IMDM medium with 5% FCS and 2% penicillin-streptomycin (Gibco). Cells were left for 7 days in the autologous mixed cellular reaction. A second stimulation was performed after 7 days and cells were again co-cultured for one week. Before and after the first and second stimulations aliquots of lymphocytes were taken to measure changes in the CD4+/CD8+ ratio by FACS analysis.

Cytometric Bead Array (CBA) Multiplex Assays

Monocytes were matured into DCs, and at day 8 supernatants of the cultured cells were collected and used for measuring cytokine production using the Cytometric
Bead Array (CBA). The analysis was done using the CBA Inflammation kit (BD), and the standard procedure was followed, as previously described. Briefly the principle of the technique is the following: different particles are labelled with a fluorescent dye which has an emission wavelength of 650 nm. Each different group of beads is labelled with such a level of fluorescent dye so that it can be distinguished by its Mean Fluorescent Intensity (MFI) by flow cytometric analysis. Beads are also coupled with antibodies that specifically recognize the molecule of interest present in the supernatant. The captured molecule is then detected through addition of a secondary fluorescent antibody. For each supernatant sample and cytokine standard mixture, 10 μl of sample or standard was added to 10 μl of the solution of the mixed bead and 10 μl of PE detection reagent. The mixture was incubated for 3 hours at RT in the dark, and afterwards data was acquired by flow cytometry using the Plate Manager ® software.

MHC-Class I restriction and IFN-γ release
To assess whether the T-cell response was MHC-Class I mediated, DCs were incubated with a MHC-Class I blocking antibody (W6/32 clone, Acris, Hiddenhausen, Germany) a method which has been previously described by Liu et al. DCs were incubated with the anti-MHC-Class I antibody for 1 hour at 37°C before the addition of lymphocytes. Lymphocytes were incubated for 5 hours at 37°C with the DCs in a 24 wells plate. The supernatants of each sample were immediately collected and used for detecting the IFN-γ release using an ELISA kit (Biosource, Camarillo, CA, USA). IFN-γ release was measured with and without inhibition of the MHC-Class I molecules by using multi-well scanning spectrophotometer (ELISA reader).

CD4/CD8 ratios before and after stimulation of lymphocytes with DCs
Before and after the first and the second incubation of lymphocytes with the electroporated DCs, lymphocytes were harvested, spun down at 1250 rpm for 5 minutes and re-suspended in FACS buffer in a concentration of 1x10^6 cells/ml. Cells were then stained in the dark for 30 minutes with the following antibodies: anti-human CD3 PE conjugated, anti-human CD4 FITC conjugated, anti-human CD8 Per-Cyp conjugated (R&D systems, Minneapolis, MN, USA); the antibodies were used in a concentration of 1:25.

Mixed Leucocytes Reaction (MLR)
The capacity of DCs to present antigens and evoke a lymphocytes response was tested in an autologous Mixed Leucocytes Reaction (MLR). Briefly, mature DCs electroporated either at monocytes or at immature DCs stage were co-cultured in flat-bottom 96-well plates (Nunc, Roskilde, Denmark) in IMDM culture medium at different target-effector ratios, starting from 4x10^3/well, down to 1.25x10^2 DCs.
(APC) per well, while each well contained a fixed number of $5 \times 10^4$ lymphocytes. After 5 days in culture, 0.2 μCi $[^{3}H]$ thymidine (Amersham Biosciences, Amersham, UK) was added to each well and the incorporation of radioactivity was measured after 16 h using liquid scintillation counting.

**MTT assay**

Before and after stimulation of the lymphocytes by the DCs electroporated either by IVT GFP-RNA or normal autologous RNA (control), an aliquot of T-cells was examined for assessing the viability rate by an MTT assay. MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann et al. is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results were read on a multi-well scanning spectrophotometer (ELISA reader).

**RESULTS**

**Lymphocyte and monocytes yields from peripheral blood**

Twelve volunteers were included, 8 male and 4 female, with a mean age of 30 (Range 24-38). Sixty four ml of blood was taken from each volunteer. The average yield of monocytes per volunteer was $20 \times 10^6$ (Range: 10 to $30 \times 10^6$). On average $60 \times 10^6$ (Range: 40 to $90 \times 10^6$) lymphocytes were obtained.

**Electroporation of monocytes with GFP-RNA and expression of GFP during the maturation of monocytes into DCs**

In Vitro Transcribed GFP-RNA was electroporated in $2 \times 10^6$ monocytes. Twenty four hours after electroporation GFP expression was seen in 62% of the gated monocytes (Fig.1B). GFP expression was measured in the GFP-RNA electroporated monocytes during eight consecutive days of culturing and maturation of the monocytes into mature DCs. GFP expression was seen at day four in 57% of the gated cells (Fig.1C), at day six, the immature DCs stage, GFP expression was seen in 55% of the gated cells (Fig.1D), and at day eight, the mature DCs stage, GFP expression was still found in 51% of the gated DCs. (Fig.1E) The maturation of monocytes into DCs was further marked by
a characteristic change in size and granularity of cells as visible in the FACS scatter plots (Fig. 2C).

**Cell differentiation and maturation markers in monocytes and DCs.**

Monocytes electroporated with GFP-RNA were matured into DCs as demonstrated by the expression levels of CD14, CD45, CD80, CD83, CD86, CD209 and CCR7 upon 8 days of maturation (Fig. 2B). Electroporated monocytes were harvested and subsequently analyzed for the expression of maturation markers 24 hours upon electroporation, at day six of culture (immature DCs) and at day eight of culture (mature DCs). Monocytes expressed CD14 in 93% and CD45 in 90% (91.4%±2.5) of the gated cells and lacked expression of CD80 (28.3%±11.2), CD83 (21%±4.2), CD86 (41%±5.5), CD209 (24%±7.01) and CCR7 (48%±4.01) (Data are expressed as mean ± SEM, Fig. 2A). After 6 days of culturing with IL-4 and GM-CSF, monocytes were differentiated into immature DCs. At this stage, CD14 was on average expressed in 15.3%±4.3 of the gated cells, and CD80, CD83, CD86, CD209 and CCR7 were expressed in 40%±4.5, 30%±5, 52%±5.8, 62%±5.1 and 10±1.5 of the gated cells respectively. At day 8, mature DCs showed CD14 expression in only
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5.2%±2 of the gated cells, but showed expression of CD80, CD83, CD86, CD209 and CCR7 in 82%±4, 80%±6.7, 90%±3.3, 83%±4.1 and 79%±6.7 of the gated cells, respectively (Data are expressed as Mean ± SEM, Fig. 2B).

**Cell loss and recovery rates after culturing and electroporation.** Viability of cells during culture and before and after electroporation procedure was determined using the Trypan Blue viability test. Mortality of monocytes

![Image](image1)

**FIG 2.** Cellular differentiation markers in monocytes and mature DCs. FACS analysis shows CD14 expression in 93% and CD45 in 90% (91.4%± 2.5) of monocytes directly after isolation and electroporation, while CD80, CD83, CD86 and CD209 were expressed in low percentages (A). In mature DCs, CD80 expression was seen in 82%±4, CD83 in 80%±6.7, CD86 in 90%±3.3, CD209 in 83%±4.1 and CCR7 in 79%±6.7 of cells, while CD14 and CD45 were expressed in a low percentage (B). The phenotypical cellular changes (shape and granularity) during maturation of the monocytes into mature DCs are visible in scatter plot profiles (C). Percentages are expressed as mean ± SEM and calculated as % of positive cells within the gated cells.
and immature DCs due to the process of culturing and recovery rates due to the process of electroporation were compared. The percentage of viable cells after six days of culturing for obtaining immature DCs was 40%±6.16. Forty eight hours after electroporation of immature DCs, performed at day six, the percentages of viable cells was 7.9±2.9% (Fig.3A). Thus from 100% of monocytes at day one, there was a cell loss of 60% due to culture, and a cell loss of 32% due to electroporation, thus in total a loss of 92%. In case monocytes were isolated from blood and directly electroporated and cultured for eight days, the percentages of viable cells at day eight (mature DC stage) was 32.13±4.7% (Fig.3B), thus there was a total cell loss of 68%. In conclusion the percentage of mature DCs as obtained from electroporated monocytes was significantly (at least three fold) higher when compared to the percentages of mature DCs obtained from electroporation of immature DCs (32%versus 8%, paired t-test, p<0.05)

**FIG 3. Quantification of DC yield comparing monocytes and immature DCs.**

Monocytes were cultured and after six days (immature DC stage) harvested and counted: the yield of immature DCs was 40%. Immature DCs were electroporated at day 6. After 48 hours of culturing, the yield of mature DCs was 8% (A). Monocytes were electroporated at day 1. After 8 days of culturing, the yield of mature DCs was 32 % (B; paired t-test, * p<0.05, n=8).
Cytokine profiles of DCs derived from electroporated monocytes and immature DCs

After electroporation of monocytes followed by eight days of maturation, GFP expressing mature DCs were obtained and supernatants were collected and analyzed for the production of IL-4, IL-5, IL-12 and IL-10 by flow cytometry using Cytometric Bead Array (BD). The same procedure was followed for the electroporated immature DCs. DCs derived from electroporated monocytes showed a significantly higher level of IL-12p70 production when compared with DCs electroporated at the immature stage (Fig. 4A; unpaired t-test, p=0.01). Simultaneously, cells electroporated at monocyte stage showed a significantly lower level of IL-10 production, when compared to cells electroporated at the immature stage (Fig. 4B; unpaired t-test, p=0.003). Other cytokines such as IL-4 and IL-5, showed no significant differences. Data are representative for the entire donor population.

FIG 4. IL-12-p70 and IL-10 production by mature DCs. FACS analysis of IL-12p70 and IL-10 production by DCs derived from GFP-RNA loaded monocytes compared to DCs derived from GFP-RNA loaded immature DCs. DCs electroporated at the monocyte stage induce significantly higher levels of IL-12p70 (A; unpaired t-tests; * p<0.05), and a significantly lower level of IL-10 when compared to DCs electroporated at the immature stage (B; unpaired t-tests *** p<0.01, n=4). Data are expressed as means ± SEM.
IFN-γ production and MHC-class I inhibition

Five hours after stimulations of autologous lymphocytes with the electroporated DCs, supernatants were collected and analyzed for measuring the IFN-γ release from lymphocytes using ELISA. This was performed before and after adding an MHC-Class I inhibiting antibody (W6/32). The basal IFN-γ release by lymphocytes was comparable to the IFN-γ release in case lymphocytes were stimulated with unloaded DCs (Fig.5). The IFN-γ release was 5 fold up in case lymphocytes were stimulated with GFP-RNA loaded DCs and increased from 0.06±0.008 to 0.32±0.007 (Fig.5; unpaired t-test **p=0.002). In case the cultures were blocked for MHC-Class I molecules the IFN-γ release by lymphocytes significantly decreased to 0.25±0.02 (Fig.5, unpaired t-test p=0.03), indicating that antigen presentation to T-cells by the DCs is MHC-Class I mediated. Data are representative for the entire donor population.

CD4+/CD8+ ratios in stimulated T-cell

Upon stimulation of the autologous lymphocytes with the electroporated DCs, CD8+ T-cell response and the proliferation of lymphocytes were measured. CD4/8 ratios of the lymphocytes were determined by FACS analysis. Before stimulation the CD4/8 ratio was approximately 3:1, after the first stimulation there was a shift towards CD8+ T-cells, the CD4/8 ratio was 2:1; after the second stimulation the CD4/8 ratio was about 1:4 (Fig.6A).
FIG 6. CD4/8 ratio, MTT assay and MLR of stimulated lymphocytes. CD4/8 ratio of the lymphocytes as measured by FACS analysis before and after the stimulation with GFP-RNA loaded monocytes matured into DCs: before stimulation the ratio was around 3:1; after stimulation, the ratio switched to almost 1:4. The MTT assay as performed on lymphocytes after two stimulations with GFP-RNA loaded DCs shows viability rate of 0.11±0.001 as a basal viability rate of the unstimulated lymphocytes, and a viability rate of 0.11±0.007 for lymphocytes stimulated by autologous normal RNA, used as control, versus 0.15±0.006 for lymphocytes which were stimulated with GFP-RNA loaded DCs (B; 0.15±0.006 versus 0.11±0.001; unpaired t-test; *** p<0.0001, n=8).

The autologous MLR of DCs electroporated at the monocytes and immature DCs stage shows that a significant increase in T-cell proliferation is induced by DCs loaded at the monocytes stage as compared to T-cell proliferation induced by DCs loaded at the immature stage (C, Paired T-test, *p<0.05, Data are expressed as Means ± SD and are representative of three different independent experiment with similar results).
MTT assay and Mixed Leukocytes Reaction (MLR)
T-cell viability was measured by MTT Assay. Lymphocytes stimulated with GFP-RNA electroporated DCs showed a viability rate of 0.15±0.006. This was 1.4 fold higher when compared to unstimulated lymphocytes which showed a viability rate of 0.11±0.001 and lymphocytes stimulated by DCs loaded with autologous normal RNA (control) which showed a viability rate of 0.11±0.007 (Fig.6B; paired t-test, p<0.0001). Simultaneously, the ability of the electroporated DCs as APC was measured performing an autologous MLR. Mature DCs electroporated with IVT GFP-RNA electroporated at the monocyte stage (day 1) induced higher T-cell proliferation as compared to mature DCs electroporated at the immature DCs stage (day 6), (Paired t-test, p<0.05, Fig. 6C). Mature DCs that were not electroporated were taken as control. Data are expressed as Mean ±SD of three independent experiments with similar results.

DISCUSSION
The possibility to isolate monocytes from peripheral blood and transform these ex-vivo into DCs, that in turn can evoke a primary immune response, has strongly enhanced the use of peripheral blood monocytes for DCs immunotherapy, particularly against several types of cancers. Although many functions of monocytes and DCs are already well known, several aspects of their complex biology still are unclear. It is known for instance that the ability of presenting antigens highly depends on the maturation state of the DCs: mature DCs are significantly more able to present antigens on their surface in order to stimulate T-cells in comparison to the immature or monocyte state, when their main ability is to process the captured antigens. A possible explanation for this phenomenon is the fact that, upon maturation, the expression level of MHC-Class I molecules increase up to a 100 fold. This theory, supported by the work of Kalady et al., suggests that DCs at best can be electroporated in their immature state when they develop the ability to process antigens. Upon maturation these DCs will be able to efficiently present the antigens to induce an immune response, for instance, via activation of cytotoxic T-cells through the MHC-Class I pathway. Therefore, the majority of studies that employed DNA or RNA for the loading of DCs, electroporated the APCs in the immature or mature state. This means that CD14+ monocytes, that are isolated from PBMCs, are first stimulated to become (CD14low) immature DCs mostly with low expression of the maturation markers such as CD80 and CD83 before performing electroporation with antigens. This two step approach in which (CD14+) monocytes first have to be cultured in a growth medium and stimulated ex-vivo to become (CD14 low) immature DCs, and than electroporated and re-cultured to be stimulated to become mature
DCs, is a laborious method, since significant amounts of cells are lost during culturing, harvesting and re-culturing. In addition, it has been observed that a considerable amount of the immature and mature DCs may be lost during the process of electroporation, although a recent study demonstrated a more efficient protocol in which fully matured DCs are more efficiently pulsed with antigens. Another general assumption is that antigens presented by monocytes may induce tolerance through stimulation of regulatory T-cells. For these reasons transfection of CD14+ monocytes has been regarded as unsuitable for inducing a cytotoxic T-cell response. Interestingly, it has been reported that monocytes directly isolated from peripheral blood and electroporated with RNA or DNA have the ability to differentiate into DCs with a mature phenotype. Yet, the immuno-potency and yield of electroporated monocytes as compared to conventional electroporation of immature DCs, and the ability to present antigens on MHC-Class I molecules in order to stimulate CD8+ T-cell clones are hardly known. Here we demonstrate an improved and efficient one step approach that results in an at least three fold higher yield of immuno-competent DCs compared to conventional methodology. In the present protocol we show that CD14+ monocytes directly isolated from peripheral blood, have significantly higher recovery after electroporation compared to immature DCs. This confirms that the tolerance of monocytes to the electroporation procedure is superior to that of immature DCs. Moreover, employing monocytes rather than immature DCs for electroporation prevents unnecessary cell loss due to culturing and harvesting of cells (Fig.3). However, it could be possible that electroporation of monocytes would change the nature of the monocytes to an extent that they would loose the capacity to become fully mature DCs. Here we demonstrated that the electroporated monocytes develop into a consistent mature DC phenotype with high expression of co-stimulatory molecules and maturation markers such as CD80, CD83 and CD86 (Fig.2B). The DCs have a high production of the important lymphocyte homing receptor CCR-7 (Fig.2B), demonstrating the capability of these cells to respond to chemokine stimulation and attraction. We showed that these cells produce sufficient amounts of the pro-inflammatory cytokine IL-12p70 (Fig.4A), and, simultaneously, the release of the immunosuppressive IL-10 was significantly lower than the IL-10 release of immature electroporated DCs (Fig.4B). We further demonstrate that the mature DCs were able to elicit a cytotoxic T-cell response. The observed T-cell response went along with sufficient IFN-γ production. Furthermore, upon stimulation of the lymphocytes with GFP-RNA electroporated DCs, the CD4/CD8 ratio shift in favour of the CD8+ T-cell clones with simultaneous increased metabolic activity, as indicated by the T-cell viability value observed in the MTT assay, which was significantly different in lymphocytes stimulated by GFP-RNA loaded DCs as compared to lymphocytes stimulated by DCs loaded by autologous normal RNA and unstimulated lymphocytes (controls), and by the induced T-cell
proliferation in the autologous MLR, where induction of T-cell proliferation was significantly higher when DCs where loaded at the monocytes stage, as compared to DCs loaded at the immature stage (Paired t-test, p<0.05). We showed a high electroporation efficacy for GFP-RNA that sustained during and after the eight days of maturation of the electroporated monocytes into mature DCs (Fig.1B-E). The persistent expression of GFP antigen during and after the maturation (Fig.1C-E) indicated that the electroporated monocytes are able to efficiently translate RNA into proteins, process antigens and express these antigens on their surface. To prove that the expression of antigens is via MHC-Class I molecules, we demonstrate that specific blocking of the MHC-Class I molecules, resulted in inhibition of IFN-γ production (Fig.5). In summary we have developed an improved and simplified method for ex-vivo manufacturing of DCs through electroporation of RNA into CD14+ monocytes directly isolated from blood. We have proved that these cells can be stimulated to differentiate into potent, fully mature DCs, highly expressing co-stimulatory molecules, DC maturation markers and CCR-7 receptor, and are able to produce high levels of pro-inflammatory cytokines. Furthermore the obtained mature DCs were able to induce an MHC-Class I mediated cytotoxic T-cell response and sustained T-cell proliferation. The currently described methodology results in an at least three fold higher yield of potent mature DCs, while redundant laborious culturing steps are eliminated. Future application of this simplified methodology may greatly enhance further developments and applications of DC RNA-based immunotherapy.

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Chapter 2

REFERENCES


Chapter 3

An ex-vivo read out for evaluation of Dendritic Cells induced autologous CTL responses against esophageal cancer

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ABSTRACT

Esophageal cancer is a highly malignant disease that despite surgery and adjuvant therapies has an extremely poor outcome. Dendritic cell (DC) immunotherapy as a novel promising strategy could be an alternative for treating this malignancy. Effective DC mediated immune responses can be achieved by raising cytotoxic T lymphocyte (CTL) response against multiple antigens, through loading DCs with total tumor RNA. However, the efficacy of this strategy first needs to be evaluated in a pre-clinical setting. The aim of the study was to set up an ex-vivo autologous human read out assay for assessing the effects of DC-mediated cytotoxic responses, using total tumor RNA as an antigen load. Biopsy specimens of seven esophageal cancer patients were used to establish primary cultures of normal and cancer cells, and to obtain autologous RNA for loading DCs. Mature DCs loaded with either normal or tumor RNA were obtained and subsequently used to raise various lymphocytes populations. Apoptosis levels of the autologous cultures were measured before and after incubating the cultures with the different lymphocytes populations. The mean apoptosis levels in the tumor cell cultures induced by lymphocytes instructed by DCs loaded with tumor RNA, significantly increased with 15.6% ±2.9 SEM (range: 3.4 to 24.5%, t-test, p<0.05). Incubation of the normal cultures with the lymphocytes populations showed a mean non significant increase in apoptosis of 0.4% ±3.4 SEM (range: -13.9 to 9.8%, t-test, p=0.7). Here we introduce a practical patient specific autologous read out assay for pre-clinical testing of DC mediated cytotoxic responses. Additionally, we demonstrated that the use of autologous tumor RNA as a strategy for raising cytotoxic responses against multiple tumor antigens could be effective for treating esophageal cancer.
INTRODUCTION

There are two major types of esophageal cancer: esophageal squamous cell carcinoma, and esophageal adenocarcinoma. For decades, the incidence of esophageal squamous cell carcinoma has been unchanged and is approximately 1 per 100,000 cases per year. Of major concern is the steadily increasing incidence of esophageal adenocarcinoma which has become an important health problem. While esophageal squamous cell carcinoma is associated with poor socio-economic status, smoking habits and alcohol intake, esophageal adenocarcinoma has a strong association with Barrett’s esophagus. Barrett’s esophagus is a metaplastic premalignant transformation of the esophageal epithelium associated with gastro-esophageal reflux disease (GERD). Although the two types of esophageal cancer have different pathophysiology, the clinical outcomes of both are poor. Even after surgical resection, the overall 5 years survival rate of these patients is less than 15%, and adjuvant treatments such as chemo- and radiotherapy have only little effect on patient outcome.

Dendritic Cell (DC) therapy, as a promising strategy to treat cancer, has been intensively investigated in the last few years. Dendritic cells (DCs) are specialized antigen-presenting cells involved in innate and adaptive immune responses. Functional DCs can be generated from human peripheral blood monocytes and be further matured into DCs that in turn can be used as vaccines for treating malignancies. To generate a cytotoxic T-cell (CTL) response against tumor cells, specific tumor antigens have to be presented to T-lymphocytes by immunactivatory DCs. Therefore, the immunogenicity of the tumor associated antigens that are used for loading the DCs is crucial. Different antigens have been used and tested for their immunopotency. These include synthetic peptides, tumor lysates, and cDNA or RNA encoding for specific tumor-associated antigens as well as total tumor mRNA. The introduction of autologous total tumor RNA as an antigen source for loading DCs has several advantages. Such a strategy will not restrict DC vaccination therapy to patients with certain HLA haplotypes. Moreover, it is most suitable to treat cancers with heterogeneous phenotypes, such as esophageal cancers and other solid malignancies that have variable expression of diverse tumor antigens. It is reasonable to assume that normal RNA that will be co-transferred with the total tumor RNA into the DCs may not result in immune responses, since there is tolerance towards self proteins through depletion of self-specific T cells. Nevertheless, when using total RNA, it is of importance to evaluate whether this strategy would induce a break in the tolerance against self-antigens. A valid pre-clinical method, which would enable us to test the efficacy of immunoactivatory DCs loaded with total tumor RNA to induce T cell reactivity in an autologous system could be of use to monitor direct adverse effects but as well to predict potential clinical responses.
The aim of our study was to create an effective autologus *ex-vivo* read out system to evaluate cytotoxic responses induced by DCs based on total tumor RNA as an antigen load. To this aim primary cultures were established from biopsies of normal and tumor tissues taken during endoscopy from esophageal cancer patients. DCs were generated from peripheral blood monocytes of the patients and loaded with either autologous normal or total tumor RNA, and subsequently studied for their immuno-stimulatory capacity. Hereupon, the DCs were used to stimulate the patient’s lymphocytes to obtain several lymphocytes populations that were subsequently tested for their cytolytic responses against the autologous primary cell cultures.

In this study we introduce a patient tailored approach using an *ex vivo* cell culture read out system for evaluating autologous DC induced cytotoxic responses. We were able to generate fully mature DCs loaded with total tumor RNA that in the autologous *ex-vivo* cultures were able to elicit cytotoxic responses specifically against the autologous cancer cells, while no significant direct adverse effect were seen against the normal cells.

**MATERIALS AND METHODS**

**Patient’s material**
The study was approved by the Academic Medical Center (Amsterdam, The Netherlands) Hospital’s Medical Ethical Committee. After informed consent and written permission, twelve patients who met the inclusion criteria (see supplementary data), were included. Before the endoscopic procedure, 64 ml of blood was drawn and collected in heparinized vials for extraction of peripheral blood mononuclear cells (PBMCs). Patients underwent endoscopic procedures for classifying, staging and grading of the esophageal cancer. During this procedure, 12 extra biopsies of each patient were taken to be used for culturing purposes and for RNA isolation; biopsies were obtained from both normal squamous epithelium taken at least 3 cm above the mass, and from the malignancy. Matching biopsies from the same spots were taken for histopathological diagnosis.

**Primary cell cultures of esophageal normal squamous epithelium and esophageal cancer epithelium**
For establishing the autologous *ex-vivo* test model, biopsies from normal esophageal epithelium and from esophageal cancer were used to establish primary cell cultures. Histological examination of the matching biopsies of the tumors showed that on estimate the biopsy specimens contained at least 50% (range 50 to 80%) of tumor cells. The culture medium MCDB 153 (Sigma) was
modified by the adding 5% fetal bovine serum, 0.4 μg/mL hydrocortisone, (Sigma), 20 ng/ml epidermal growth factor (GIBCO, Grand Island, NY), 10⁻¹⁰ mol/L cholera toxin (Sigma), 140 μg/mL bovine pituitary extract (Sigma), 20 μg/mL adenine (Sigma), 100 U/mL penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), 0.25 μg/mL amphotericin B (GIBCO), 5 μg/mL insulin-transferrin (GIBCO) and 4 mmol/L glutamine. The explant method was as described before. Briefly, biopsies from normal and tumor esophageal mucosa were collected aseptically into MCDB153 modified medium during routine endoscopy of patient with esophageal cancer. Specimens were processed within half an hour of procurement as follows: biopsy specimens were minced into fragments of 1-2 mm³ in size. The pieces of tissue were placed in a 24 wells plate and anchored by a sterile glass microscope slide before adding of growth medium. One ml/well MCDB 153 modified medium was added and cultures were placed at 37°C and 5% CO₂. Fresh medium was replaced every three days for three weeks until measurement of autologous cytotoxic responses.

RNA isolation
Biopsies from normal and cancer tissues were collected in Trizol Reagent (Life Technologies Inc, Invitrogen, Breda, The Netherlands) and processed according to the manufacturer’s instructions. Briefly, tissues were lysed by adding 200 μl Trizol. After phenol/chloroform extraction, RNA was precipitated with isopropanol, washed with 70% ethanol and air-dried. The RNA was then dissolved in RNase-free H₂O and stored at -80 °C until required. One μl of total RNA was used to quantitate the RNA by spectrophotometry using the Nanodrop® apparatus (type ND-1000, Wilmington, USA).

Isolation of lymphocytes and monocytes from peripheral blood of the patient
Lymphocytes and monocytes were isolated from 64 ml of peripheral blood collected from patients in heparinized vials, using the Ficoll-Percoll gradient separation method. A first separation of peripheral blood mononuclear cells (PBMCs) was done using Ficoll-Hypaque solution (Amersham, Pharmacia, Piscataway, NJ, USA). A further separation of monocytes from lymphocytes was done using a Percoll (Amersham Biosciences Europe Freiburg, Germany) density gradient separation, as described previously. Briefly, PBMCs were washed in Roswell Park Memorial Institute (RPMI) 1640 medium (BioWhittaker-Cambrex Bioscience, Walkersville, MD, USA) at 1500 rpm for 5 minutes twice. At the mean time, 19.8 ml of Percoll were mixed with 2.2 ml 10x Phosphate Buffered Saline (PBS) to obtain Standard Isotonic Percoll solution (SIP), and then with Iscove’s Modified Dulbecco’s Medium (IMDM) medium (BioWhittaker), to obtain three solutions at different concentrations (60, 47.5 and 34% SIP). PBMCs were then re-suspended in 2.5 ml of 60% SIP, then 5 ml of 47.5% SIP and 2 ml of 34% SIP.
were added and a centrifugation at 3100 rpm for 45 minutes was performed. After centrifugation, the upper layer (monocytes) was collected as well as the lower layer (lymphocytes). Once separated, monocytes and lymphocytes were washed twice in IMDM, and either used immediately or cryo-preserved in a Dimethylsulfoxide (DMSO)/Fetal calf serum (FCS) (2:8) solution (Merck, Darmstadt, Germany; GIBCO BRL, Grand Island, NY, USA, respectively).

**Electroporation of monocytes with normal and tumor autologous total RNA**

Monocytes characterized as CD14+, CD83-, CD86-, CD209- were used for electroporation with autologous total normal or tumor RNA following the procedure recently described by Milano et al.\(^5\). To monitor for the electroporation efficacy, In Vitro Transcribed (IVT) GFP-RNA was used. The procedure was as follows: monocytes freshly isolated from blood were washed twice in IMDM and electroporated using the Amaza cell line Nucleofector Kit V (Amaza GmbH, Cologne, Germany): 0.5 up to 1x10\(^6\) cells were mixed with Cell Line Nucleofector solution V, and 5 µg/ml of RNA was added to the cuvette to be electroporated using the Nucleofector program U16 of the Amaza Nucleofector device. After electroporation, 1 million/ml monocytes were cultured in 24 wells plate using IMDM with 10%FCS and 2% penicillin/streptomycin (GIBCO) and cells were placed at 37°C and 5% CO\(_2\) for one hour, then cells were washed twice with IMDM, and 1000 U/ml of IL-4 and 800 U/ml GM-CSF were added to initiate the maturation process. After six days at the stage of immature DCs, the cytokines IL-1β, TNF-α and LPS (10 ng/ml, 25 ng/ml, 0.02 ng/ml respectively) were added to further enhance the maturation process. Cells were analysed for the expression of maturation markers at day six and eight by FACS.

**Detection of markers and MHC class I and II in monocytes and DCs**

Monocytes were harvested by pipetting, washed and re-suspended in FACS buffer (5 g BSA, 0.1 g NaN3, 100 mM EDTA in 1 L PBS) in a concentration of 1 million cells/ml, then incubated with various fluorochrome-conjugated antibodies on ice for 30 minutes in the dark, then washed again and analyzed using the FACSCALIBUR apparatus (Becton-Dickinson, Franklin Lakes, NJ, USA) and BD CellQuest Pro software. PE or FITC conjugated antibodies, specific for CD14, CD83, CD86, CD209, CCR7, HLA-A,B,C and HLA-DR (BD, San Jose, CA, USA) were used as appropriate, in a concentration of 1:25. Sample stained for FITC - and PE-conjugated IgG2a/IgG, Isotype controls (BD Biosciences) were included in the staining procedure. For all cases markers and MHC-Class I and II expression were measured during maturation from monocytes, to immature and mature DCs. Measurements were performed of both electroporated and not electroporated (control) cells.
Stimulation of autologous lymphocytes with electroporated DCs
After eight days from the isolation of monocytes, the resulting mature DCs were twice co-incubated for seven days with autologous lymphocytes in a proportion of 1:4 (5x10⁵ DCs and 2x10⁶ lymphocytes) in 24 wells plate in IMDM medium with 5% FCS and 2% penicillin-streptomycin (GIBCO). Before and after the first and second stimulation (day 1, day 7 and day 14 of the co-culture) aliquots of lymphocytes were taken to measure changes in the CD4/8 ratio by flow cytometry and the supernatants were collected to measure production of inflammatory cytokines. Finally, per patient two populations of lymphocytes were obtained: lymphocytes stimulated by DCs electroporated with tumor RNA and a second population of lymphocytes stimulated by DCs electroporated with normal RNA.

Measuring of the cytotoxic responses on the ex-vivo cell cultures
After stimulation with the normal and tumor RNA electroporated DCs, the two different populations of lymphocytes mentioned above were washed in IMDM and added to the autologous cultured normal and tumor epithelial cells at a target/effect ratio of 2x10⁴/4x10⁵ cells for 5 hours. For each patient autologous tumor and normal epithelial cell cultures were incubated with lymphocytes either stimulated by DCs loaded with autologous normal RNA or with autologous tumor RNA (see supplementary picture). The epithelial normal and tumor cells were washed with cold PBS and detached by adding 0.5ml trypsin (GIBCO, Auckland, NZ) for five minutes at 37°C; cells were then collected in 1 ml MCDB 153 modified medium, spun down and re-suspended in Annexin V buffer (2.38g HEPES, 8.8g NaCl, 0.38g KCl₂, 0.2g CaCl₂, 0.20 g MgCl₂) in a concentration of 1 million cells/ml. Apoptosis of the normal and cancer cells was measured by using the following antibodies: anti-human Annexin-V APC conjugated (ICQ, Groningen, The Netherlands); Via-probe 7AAD (necrosis marker; R&D System); anti-human EpCam FITC conjugated (epithelial specific marker; Miltenyi Biotech, Auburn, CA); anti-human CD3 PE conjugated (T-cells marker; R&D System). Data were acquired using BD Cell Quest Pro Software. Apoptotic epithelial cells were gated as double positive for AnnexinV and EpCam, and negative for CD3 and 7AAD.

Cytometric Bead Array (CBA) Multiplex Assays
During stimulation of lymphocytes with normal and tumor RNA electroporated DCs supernatants were collected of the samples of the patients at day 7 (after the first stimulation) and at day 14 (after the second stimulation) and analysed for cytokine contents using the Cytometric Bead Array (CBA). Experiments were performed using the CBA Inflammation kit (BD) following the manufacturer’s instructions, and the standard procedure was followed as previously described³⁰. Acquired data were analyzed using the BD calibration and analysis software.
Statistical analysis
Statistical analyses were performed using the software Graph Pad Prism®. Statistical test were applied to 7 independent experiments. Differences between the values were determined using both Student’s paired t-test and one way ANOVA. Significance was determined as P<0.05.

RESULTS

Patients and primary cultures
A total of twelve patients were enrolled in the study. Informed consent was obtained from each participant. Because of difficulties in establishing primary cultures, such as a high rate of apoptosis due to the initial state of certain tissues and bacterial contamination, a final number of seven male esophageal cancer patients with a mean age of 68 (range 61-78), were analyzed for autologous CTL responses. Histopathology of the biopsies of the cancers was determined by an expert pathologist and revealed esophageal adenocarcinoma in five patients, and squamous cell carcinoma in two patients. Patient data are summarized in table 1.

Table 1: Data of patients included in the study

<table>
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<td>T3N1M0</td>
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Immuno-phenotyping of mature DCs
The immuno-phenotype of DCs was analyzed by FACS. The percentages of gated positive cells for the markers CD14, CD83, CD86, CCR7 and CD209 were as follows: at day one, at the monocyte stage, on average 95% of the gated cells were positive for CD14, whereas all the other markers were expressed in less than 20% of cells. At the immature stage, CD83, CD86, CD209, CCR7 expression was seen on average in 50 to 60%, while CD14 expression decreased to 20%. After
further two days maturation, the percentage of CD83, CD86, CD209 and CCR7 expression increased to at least 90%, while CD14 decreased on average to less than 20% of the gated cells (Fig 1A). Expression of these markers was comparable between electroporated and not electroporated (control) cells, indicating that the process of electroporation did not influence the maturation process.

Figure 1: Cellular differentiation markers in monocytes, immature DCs and mature DCs.

A: FACS profiles of the cellular differentiation markers: CD14, CD86, CD83, CD209 and CCR-7, after electroporation in mature DCs: CD14 expression is seen in less than 20% of the cells, while the maturation markers are found in at least 90% of the gated cells. B: Electroporation efficacy assessed by FACS analysis shows high levels of GFP expression of DCs loaded with IVT GFP-RNA, after 72 hours and at day eight of maturation. Bright grey lines represent control cells; dark grey areas represent the positive cells.
Electroporation efficacy as measured by GFP expression

The electroporation efficacy as monitored by electroporation of IVT GFP-RNA, showed GFP expression in 50 to 60% of the gated cells in all cases (Fig. 1B). This expression was found 24 hours after electroporation and was maintained during the maturation process of the DCs until day eight.

MHC-Class-I and II expression

MHC-Class-I was seen on average in 90% of the gated cells in the monocyte stage. At day six (immature DC state), 90% of cells maintained expression and at day eight (mature DC state) MHC-Class-I increased to 95% (Fig.2).

MHC-Class II was seen on average in 90% in the monocyte stage. At day six (immature DC state) the expression level was retained, and at day eight, (mature DC state), MHC-Class II expression increased to 96% of the gated cells (Fig.2). No significant differences were detected between the expression levels of electroporated and not electroporated (control) cells.

Figure 2: MHC-Class I and II expression before and after transfection of the DCs. FACS analysis of MHC-Class I and II (HLA-A, B, C and HLA-DR expression) in monocytes before and after electroporation, at the immature stage (day 6) and mature stage (day 8) and in not electroporated mature DCs. Before electroporation the MHC class I and II expression is seen on average in 90% of the gated cells. The expression levels of both MHC-Class I and II molecules is maintained through the maturation process and it is still high (on average 90%) in mature DCs. Bright grey lines represent control cells; dark grey areas represent positive cells.
CD4/8 ratios in lymphocytes

Before stimulation the CD4/8 ratio was 2.5±0.1 SD (Fig.3). After one week of stimulation by DCs electroporated with normal RNA, lymphocytes showed a ratio shift to 2.2±0.9 SD and to 2.1±1.1 after the second week of stimulation. Thus, there was no significant difference in the CD4/8 ratio in the lymphocytes stimulated before and after stimulation by DCs loaded with normal RNA (two tailed paired t-test, p=0.8). In contrast, after one and two weeks of stimulating the lymphocytes by DCs electroporated with tumor RNA, the ratio shifted to 2.1±1.2 SD, and to 0.7±0.3 SD, respectively, which was a significantly different shift (Two tailed paired t-test, **p=0.006).

IFN-γ production by stimulated lymphocytes

Before stimulation IFN-γ production of the lymphocytes was virtually negative. After stimulation with not electroporated DCs or DCs electroporated with normal RNA, the IFN-γ level increased to 2041 ±650 SEM and 2425 ±294 SEM pg/ml respectively. After stimulation of the lymphocytes by DCs electroporated with tumor RNA, the IFN-γ production significantly increased to 5742 ±592 SEM pg/ml (ANOVA, Bonferroni multiple comparison post test, ***p<0.001; Fig 4).

Figure 3: CD4/8 ratios of stimulated lymphocytes.
CD4/8 ratios of lymphocytes as measured by FACS: The CD4/8 ratio of the lymphocytes before stimulation as measured in all cases was 2.5±0.1 SD (A, B). A: After the subsequent stimulation of the lymphocytes by DCs electroporated with normal RNA, the CD4/8 ratio shifted to 2.2±0.9 SD; and after the second stimulation to 2.1±1.1 SD (Two tailed paired t-test, p=0.8). B: Lymphocytes stimulated by DCs electroporated with tumor RNA showed a CD4/8 ratio of 2.1±1.2 SD after the first week of stimulation, and 0.7±0.3 SD after the second stimulation (Two tailed paired t-test, **p=0.006).
Figure 4: IFN-\(\gamma\) release by lymphocytes stimulated with normal and tumor RNA electroporated DCs. IFN-\(\gamma\) release as measured in lymphocytes before and after stimulation with electroporated DCs. Lymphocytes stimulated by DCs electroporated with tumor RNA show a significantly higher IFN-\(\gamma\) release than lymphocytes stimulated with not loaded DCs or lymphocytes stimulated by DCs electroporated with normal RNA. (ANOVA, Bonferroni multiple comparison post test, \(*\*, p<0.001\)). Data are expressed as means ± SEM of the different experiments.

Figure 5: Apoptosis levels (Annexin V expression) measured by FACS. FACS analysis of 7AAD and AnnexinV (apoptosis) expression in the normal and tumor primary cell cultures of patient one. Basal level of apoptosis in normal primary cultures (A); apoptosis in normal primary cultures after incubation with lymphocytes stimulated with DCs loaded with either normal RNA (B) or tumor RNA (C); Basal level of apoptosis in primary tumor cultures (D); apoptosis in primary tumor cultures, after incubation with lymphocytes stimulated by DCs loaded with normal RNA (E) and tumor RNA (F). Notice that a high level of apoptosis was induced in the tumor cultures by lymphocytes stimulated with tumor RNA loaded DCs (F), while there is no increase in apoptosis in case the tumor cells were incubated with lymphocytes stimulated by DCs loaded with normal RNA (E). Microphotographs of the normal epithelial cultures and tumor epithelial cultures are included in the figures and were obtained using an Eclipse TS 100 phase contrast microscope (NIKON).
Cytotoxicity responses as measured by apoptosis in the epithelial cell cultures

Apoptosis levels after 5 hours of incubation with the cytotoxic populations as calculated with respect to the basal apoptosis levels of the cultures (Fig. 5A,D), were as follows: After incubation of the autologous normal epithelial cell cultures with lymphocytes stimulated by DCs electroporated with normal RNA and tumor RNA, the mean increase in apoptosis levels were 0.4% ±3.4 SEM (range: -13.9 to 9.8%) and -1.3% ±3.2 SEM (range: -14 to 13.4%) when incubated with lymphocytes instructed by DCs loaded with normal and tumor RNA, respectively (Two tailed t-test, p=0.7 and 0.2). (B) On average the apoptosis levels in the tumor cell cultures incubated with lymphocytes instructed by DCs loaded with tumor RNA, increased with 15.6% ±2.9 SEM (range: 3.4 to 24.5%, ** p=0.002), while with 2.3% ±2.1 SEM (range: -4.7 to 12.7%), when incubated with normal RNA electroporated DCs (p=0.2).

Figure 6: Summary of the CTL induced apoptosis levels in the primary ex-vivo cultures. Basal level of apoptosis in the normal and the tumor epithelial cells was taken as zero. (A) On average, the level of apoptosis in the normal epithelial cultures of the patients increased with 0.4%, ±3.4 SEM (range: -13.9 to 9.8%) and -1.3% ±3.2 SEM (range: -14 to 13.4%) when incubated with lymphocytes instructed by DCs loaded with normal and tumor RNA, respectively (Two tailed t-test, p=0.7 and 0.2). (B) On average the apoptosis levels in the tumor cell cultures incubated with lymphocytes instructed by DCs loaded with tumor RNA, increased with 15.6% ±2.9 SEM (range: 3.4 to 24.5%, ** p=0.002), while with 2.3% ±2.1 SEM (range: -4.7 to 12.7%), when incubated with normal RNA electroporated DCs (p=0.2).
Chapter 3

5E, Fig.6). The mean level of apoptosis in the tumor cell cultures incubated with lymphocytes instructed by tumor RNA electroporated DCs increased significantly with 15.6% ±2.9 SEM (range: 3.4 to 24.5%, Two tailed paired t-test, p<0.05 Fig.5F, Fig.6B).

DISCUSSION

Clinical trials using antigen-pulsed DCs have been conducted in patients with various types of cancer, including myeloid leukemia, glioblastoma, metastatic melanoma, pancreas, colorectal cancer, and many others. To our knowledge, no reports have been yet published regarding DC therapy on esophageal cancer. Genotypically and phenotypically, esophageal cancers are highly heterogeneous, which makes it difficult to choose a single effective antigenic target for treatment of these type of malignancy. Using total tumor RNA as an antigen load for DCs overcomes the need to identify specific tumor antigens, but as well provides the opportunity to evoke more effective anti-cancer cytotoxic T cell responses against tumors without knowing the exact nature of the targeted antigens. RNA-based DC immunotherapy with the use of total tumor RNA provides the potential to generate a polyclonal immune response to multiple known and unknown tumor antigens without the limitation to specific HLA types. However, when using total tumor RNA for loading DCs there is a major concern for inducing direct adverse effects through breaking tolerance to self antigens resulting in autoimmune responses. Therefore, a preclinical ex-vivo evaluation in an autologous set up to estimate potential adverse effects of this strategy could be useful to predict inauspicious effects in a clinical setting.

In our study we analyzed the feasibility of an autologous read out system to evaluate cytotoxicity responses against esophageal cancer cells. Furthermore, in this study, the concern of breaking tolerance to self-antigens, when immunizing with total tumor RNA, was addressed by monitoring lysis activity against the patient’s normal epithelial cells in the ex-vivo read out system. In general we found that DCs electroporated with tumor RNA elicited cytotoxic lymphocytes which were able to recognize and induce significantly high levels of apoptosis in the autologous cultured cancer cells (p<0.05, Fig.6), while these lymphocytes did not induce a cytotoxic reaction against the autologous normal epithelial cells (p=0.2, Fig.6). In addition, in the control experiments with lymphocytes stimulated by DCs loaded with normal RNA, in general no significant increase in apoptosis levels in the autologous normal epithelial cells was detected. This observation corresponds with a previous study, in which DCs electroporated with renal tumor RNA, did not show a cytotoxic response against benign renal parenchyma. It needs to be pointed out, however, that the increase in level of
apoptosis in the normal cultures ranged from -13.9 to 12%. Thus, in certain cases this could mean that the DCs can mediate adverse effects on normal tissues. In one particular case, for instance, the DCs loaded with tumor RNA induced a cytotoxic lymphocytes mediated increase in apoptosis level of 12% in the normal cells and, although this was significantly lower when compared to the apoptosis level of 22% as induced in the tumor cells (p<0.05, Fig.6), it would be doubtful whether or not these DCs would be suitable as a vaccine for patient therapy.

Despite the strong potency of DCs to present tumor antigens, the efficacy of therapeutic DC vaccination against cancer is questioned. So far only a limited rate of objective tumor regressions have been observed in clinical studies. One reason for the discrepancy between the outcomes as seen in the preclinical feasibility assays with respect to the true clinical responses could be that most preclinical *in vitro* tumor models to evaluate direct cytotoxicity of DCs have been performed on either cancer cell lines or mice models. Because of an absent or different immune system, tumor cell lines and animal models in general will exhibit remarkable anticancer responses, while these models are not suitable to predict direct adverse immune responses in humans. Indirectly, the potency of DCs is measured through their ability to induce pro-inflammatory cytokines such as IL-12, for their migratory ability, their potency to induce allogenic T-cell responses and tumor antigen-specific CD8+ T cells. Therefore, another potential application of the test model is its application for predicting clinical anti tumor responses. In our seven cases cytotoxic lymphocytes induced apoptosis was on average 15% in the autologous tumor cultures, but the range of lysis was as low as 4% and as high as 24%. It is possible that these *ex-vivo* responses would correlate with clinical responses, and as such this set up might be used as a prognostic clinical tool. Unquestionably, this should be first evaluated in patient trials, during which as well late adverse responses can be monitored and long term safety of total tumor RNA based DCs can be studied. Of interest is a similar recent study, which demonstrated that DC induced preclinical *ex-vivo* CTLs responses on autologous tumor cell cultures of melanoma patients do correlate with clinical tumor responses.

The main difficulty of our set up is the loss of the cultures due to bacterial overgrowth and cell death. Five of twelve cultures were lost because of these reasons. This is not surprising since esophageal cancers are highly contaminated and may contain necrotic areas. The majority of cultures were lost in the initiation phase of the study. Optimizing the culturing media, targeting biopsies from non necrotic tumor areas and minimizing the delay between collection of the tissues and setting up of the cultures highly diminished the number of losses.

In this study loading of the DCs was performed through direct electroporation of the monocytes with total RNA. This method that has been recently described was found to be particularly efficient with a high yield of fully mature immuno-potent
Furthermore, the method used did not interfere with the expression of the most important maturation markers, such as CD83, CD86, CD80, CD209, the expression of MHC-Class I, which is of pivotal importance for a proper CD8+ T cell priming, and MHC-Class II, of central importance for inducing CD4+ T helper response (Fig.2).

Another important finding in the present study is that the immuno-potency of the DCs loaded with tumor RNA was significantly higher compared to those loaded with normal RNA. This was demonstrated by a significantly more profound shift of the CD4/8 ratio towards CD8+ T cells (Fig. 3), and the 3 fold increase in the level of IFN-γ (Fig. 4). The more significant shift towards a CD8+ T cells subpopulation induced by tumor RNA loaded DCs on autologous lymphocytes as compared by the shift induced by normal RNA loaded DCs, indicates that lymphocytes stimulated by tumor RNA loaded DCs were primed towards a cytotoxic population, whereas lymphocytes stimulated by normal RNA loaded DCs maintained tolerance for self antigens and as consequence the shift towards CD8+ T cells was not significant. This hypothesis is further sustained by the additional shift towards CD8+ T cells after the second stimulation of lymphocytes that was only seen in case DCs were electroporated with tumor RNA. This suggests that lymphocytes that were primed in the first stimulation, further expanded after the additional stimulation with tumor antigen presenting DCs.

In summary, here we present a human autologous ex-vivo read out system that appears to be an effective and useful read out for testing the efficacy of DC induced cytotoxic responses against esophageal cancer cells, and to monitor for direct adverse effects on autologous normal tissues of esophageal cancer patients. In addition, the minimal lytic effects on the normal cell cultures encourages the use of total tumor RNA based DC vaccines. This important ex-vivo validation study sets the stage to proceed with establishment of this novel model for preclinical testing of CTLs and antigen presenting cells, and to further explore the strategy of total tumor RNA based DC therapy for treatment of esophageal cancer.
REFERENCES


Chapter 4

Characterization of esophageal cancer microenvironment: a balance towards tumor or towards anticancer immune-response?

Francesca Milano, Tineke Jorritsma, Agnieszka M. Rygiel, Jacques J.G.H.M. Bergman, Carine Sondermeijer, Maikel P. Peppelenbosch, Anja Ten Brinke, Marieke S. van Ham, Kausilia K. Krishnadath

Submitted
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ABSTRACT

OBJECTIVE: Immunotherapy of esophageal adenocarcinoma (EAC) remains disappointing, possibly because of a therapy-unfavorable tumor microenvironment, prompting investigations as the nature of this environment.

DESIGN AND PATIENTS: Biopsies of tumor and normal tissues were collected from 17 EAC patients, and investigated using fluorescent immunohistochemistry (IHC) for COX-2, VEGF, TGF-β, IDO, CXCL3 and the receptor CXCR1 expression, or for measuring cytokine levels by cytometric bead array (CBA) and quantitative PCR (QPCR).

RESULTS: IHC of normal and tumor samples showed that COX-2, VEGF, TGF-β, CXCL3, CXCR1, and IDO expression is up-regulated in varying degrees in the tumor biopsies in 80% to 93% of the patients, whereas QPCR revealed that the cytokine IL-8 is significantly up-regulated in 58% of the EAC cases. CBA confirmed high IL-8 expression in 80% of the tumors (Mann-Whitney test, p<0.05), and high IL-1β in 86% of the tumor tissues as compared to normal tissues (p<0.05). IL-6 was only detectable in 10% of tumors, and IL-10, IL-12, IL-4 and IFN-gamma were detected neither in the tumor nor in the normal tissues.

CONCLUSIONS: The EAC microenvironment is characterized by a lack of cytokines that normally would enhance anti-cancer responses, such as IFN-γ and IL-12, and by a high expression of several immuno-suppressive cytokines, and a wide range of tumor growth promoting factors, such as COX-2, VEGF, TGF-β and IL-8. For future treatment of patients with immunotherapies it will be important to specifically target these factors and combine immunotherapy with immune modulating agents in order to improve treatment efficacy.
INTRODUCTION

Dendritic Cell (DC) therapy for various types of cancer has received considerable attention the last decades, and proof of principle that DC vaccination is an appealing strategy to treat cancer has been obtained on multiple occasions. Nevertheless, the limited rate of objective tumor regressions observed in clinical studies, have raised questions as to the actual efficacy of therapeutic vaccination against cancer in a practical setting\textsuperscript{1-3}. The reasons underlying the relatively poor efficacy of DC vaccination remain only partially understood\textsuperscript{4}.

Among the often-cited factors hampering effective anti-cancer immunotherapy, the immuno-suppressive network created as a consequence of pathological interactions between cancer cells and host immune cells features prominently\textsuperscript{5-7}. It has become clear that it involves a multitude of factors whose production is under control of a complex interplay between the normal host epithelial cells, invading tumor cells, stromal fibroblasts, inflammatory cells, proliferating endothelial cells, an altered extracellular matrix, and growth factors activating oncogenic signaling pathways\textsuperscript{8}. This altered tumor environment will result in altered production of for instance co-stimulatory and co-inhibitory molecules, such as the cytokines IL-6, IL8, IL-1β, TNF-α, IFN-γ, IL-12, IL-4 and many others, and an imbalance of effector T- and regulatory T-cells (Tregs)\textsuperscript{9} finally resulting in tumor immune evasion\textsuperscript{10,11}.

Although in vivo data are still largely lacking, a number of interesting factors, which may participate in tumor-permissive micro-environments have now been identified. Maintaining tumor growth requires an efficient supply of nutrients and oxygen, therefore pro-angiogenic factors, like vascular endothelial growth factor (VEGF)\textsuperscript{12} and the CXCR/CXCR ligand axis including IL-8 (CXCL8)\textsuperscript{13,14} seem important factors, and may exert immunosuppressive actions as well. In addition, it has been demonstrated that production of VEGF profoundly inhibits maturation of DCs, which are major inducers of effector T-cell proliferation\textsuperscript{15}. Generally considered as a potential more important factor is the expression of COX-2, which provokes strong immune-suppression through the production of PGE\textsubscript{2}\textsuperscript{16}, and subsequent diminished IL-12 release by mature DCs, in turn impairing Th1 responses\textsuperscript{17}. Several studies have demonstrated that COX-2 expression is strongly correlated with poor prognosis\textsuperscript{18} and it is assumed that inhibition of this protein would induce down-regulation of important oncogenic pathways\textsuperscript{19,20}. In addition, another factor, which can contribute to the immune-suppression and inhibition of T-cell proliferation and attracts substantial attention is Indoleamine 2-3 dioxygenase (IDO), a tryptophan-catabolizing enzyme, which inhibits T-cell proliferation. Munn et al. have previously described a subset of human monocyte-derived DCs that can induce T-cell inhibition by excretion of IDO\textsuperscript{21,22}. Finally, some authors note that IL-1β, together with TNF-α are known to induce production of IL-6, which in turn mediates the inhibition of an appropriate
DC differentiation and may also be involved in providing the tumor with a favorable environment.

Apart from the active expression of immunosuppressive factors, inhibition of production of cytokines that induce DC differentiation, such as IL-12 and IFN-gamma, may also induce tumor immune-tolerance. It may be predicted that an impaired balance in immune-stimulatory and immune-suppressive factors leads to defects in adaptive immune responses, and in particular may impair anti-tumor DC functioning, partly explaining the limited success obtained hitherto with DC vaccination studies of solid cancer and prompting investigation to clarify whether such a disturbed balance is present in patients. It is important to stress, however, that a clear demonstration that such a tumor micro-environment exists in esophageal adenocarcinomas (EAC) patients has not been clearly provided yet.

Since EAC are highly malignant and seem to be resistant to conventional therapies such as chemo- and radiotherapy, we anticipated that EAC patients may highly benefit from DC immunotherapies in the future. EAC is associated with Barrett’s esophagus (BE), a metaplastic condition of the distal esophagus, in which after gastro-esophageal reflux injury, the normal squamous epithelium is replaced by columnar epithelium. These cancers have an extremely poor prognosis, with an overall five years survival rate of less than 15%. The aim of our study was to gain further insight in the EAC tumor microenvironment, in order to find novel targets for developing immunotherapies and for more effective combinatorial treatment strategies. In order to achieve this, we performed immunohistochemistry (IHC) on normal and tumor EAC biopsies, staining for several factors, such as COX-2, VEGF, TGF-beta, IDO, and CXCL3 and its receptor CXCR1. Furthermore we isolated RNA from biopsies and perform quantitative PCR to establish the balance between pro-inflammatory and anti-inflammatory cytokines, such as IL-6 and IL-8. To confirm the presence of the cytokines analyzed by PCR, we obtained lysates of biopsies and measured the cytokine levels by Cytometric Bead Array.

Our results show that the EAC microenvironment is characterized by a disturbed immunological background specifically characterized by high levels of IL-8 and IL-1β, while other immunosuppressive factors such as IL-6 and IL-10, are not found in EAC. Definition of the immunological microenvironment in EAC provides us with rational clues for the future treatment of this disease.

MATERIALS AND METHODS

Patient material and characteristics
After informed consent and written permission, 17 EAC patients were included. Patients underwent endoscopic procedures for classifying, staging and grading of the esophageal cancer. During this procedure, 12 extra biopsies, 6 from normal
and 6 from tumor tissues, of each patient were collected and immediately stored at -80°C to be used for RNA isolation, IHC, and for obtaining lysates for protein measurements; biopsies were obtained from normal squamous epithelium, taken at least 3 cm above the visible mass, and from the malignancy. Matching biopsies from the same spots were taken for histopathological diagnosis.

### Table 1

<table>
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<td>T1N0M0</td>
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<td>77</td>
<td>male</td>
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</tr>
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**RNA isolation**

Total RNA for PCR purposes was isolated from normal squamous and tumor biopsies. For each biopsy, 300 μl of Trifast (Peqlab Biotech, GmbH, Erlangen, Germany) were mixed with 3μl of glycogen, samples were vortex for 10 seconds, then 60 μl chloroform was added and again the samples were vortex for 30 seconds. A centrifugation step of 10 minutes at 13000 rpm at RT followed, then the supernatants were collected into a fresh tube and 180μl isopropanol was added and samples vortex for 30 seconds. The RNA was precipitated overnight at -20°C. The next day, a centrifugation step of 30 minutes at 13000 rpm at 4°C was performed, then the supernatant was discarded and the RNA pellet was washed with 75% ethanol. The pellet was air-dried, and then dissolved in 16.5 μl DEPC water (Ambion, Nieuwerkerk a/d, Nijssel, The Netherlands). In order to dissolve the RNA completely, the tubes were placed at 55-60°C for 3 minutes,
vortexed and spun for two times. Upon isolation, 1.5μl of RNA solution was used to determine purity and concentration using Nanodrop® (type ND-1000). Assessment of the quality was performed with the RNA 2100 Pico Labchip kit (Agilent Technologies, Amstelveen, The Netherlands) using 1μl of total RNA.

**Quantitative PCR (Taqman)**

Preparation of cDNA: to each RNA sample, 2μl of random Hexamers were added, and then incubated for 10 minutes at 70°C. Tubes were cooled on ice, then 15μl of RNA was mixed with the following: 10μl 5X RT-buffer, 5μl 0.1M DTT, 10μl 10mM dNTPs, 0.5μl RNase inhibitor, and 0.5μl of Superscript RT (200U/μl). Samples were incubated for 10 minutes at RT, then 50 minutes at 42°C, and 15 minutes at 70°C. cDNA was obtained for quantitative RT PCR using Taqman 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Real time PCR was performed using primers for selected genes which were developed, such as for IL-8, IL-6 and 18S RNA as control (Table 2). Reactions were performed in 20μl total volume, consisting of 5μl of sample diluted 1:50, and 15μl Master Mix constituted by PCR Master Mix SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) , and DEPC water (Ambion). Each sample was analysed in duplicate. The thermal Cycle conditions were: Cycle Time Temperature Repeat Ramp Time, 2’ at 50°C, hold 10’ at 95 ºC, [(cycle 15” at 95 ºC, 1’ at 60 ºC )x 40], [(15”20 ºC , 1’ at 95 ºC )x1]. The level of gene expression within each sample was adjusted to levels of an internal control (human ribosomal 18S RNA) before expression was calculated as a percentage of the level of gene expression using the control samples.

**Table 2**

<table>
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<tr>
<th>Cytokines</th>
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<td>IL-8</td>
<td>F: TTGGCAGCCTTCCTGATTTC&lt;br&gt;R: AACTTCTCCACAACCTCTG</td>
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<tr>
<td>IL-6</td>
<td>F: GTACATCCTCGACGGCATC&lt;br&gt;R: CCAGGCAAGTCTCCTCATTG</td>
</tr>
<tr>
<td>18S-rRNA</td>
<td>F: CGGCTACCATCCGAGGAA&lt;br&gt;R: GCTGGAATTACCGGGCT</td>
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</table>

**Fluorescent immuno-histochemistry of normal and tumor biopsies**

Tissue sections from squamous and EAC patients were embedded in Tissue-Tek®, Optimum Cutting Temperature compound (Sakura Finetek, USA, Torrance, CA) at -20°C, and sectioned using a cryo-microtome (Microm HM 550) in serial cryostat sections of 6μm, placed on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides and air-dried overnight. For routine histological examination, of each block one slide was routinely stained by Hematoxylin and Eosin (H&E). Slide preparations were fixed for 20 minutes.
in Phosphate Buffered Saline (PBS) with 4% Paraformaldehyde (PFA) and 0.1% Triton X-100 and washed in PBS. Blocking of aspecific antigens was performed by incubating slides for 45 minutes with PBS with 1% Bovine Serum Albumin (BSA) with 10% Fetal Calf Serum (FCS). Slides were washed with PBS and incubated overnight at 4°C with the appropriately diluted primary antibody in PBS with 1% BSA and 0.1% Triton X-100. After incubation the slides were washed with PBS and incubated with the secondary FITC conjugated antibody as appropriate (Dako, Glostrup, Denmark) 1:1000 diluted in PBS. Slides were washed and mounted with DAPI (Roche, Mannheim, Germany)/Vectashield (Vector laboratories Inc, Burlingame, CA, USA) 1:1000. The antibodies used and dilutions are summarized in Table 3.

A single consultant pathologist reviewed all the H&E stained tissue sections.

### Table 3

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Company</th>
<th>Country</th>
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</tr>
<tr>
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### Analysis of the IHC results

For each patient, two different biopsies of tumor were compared to two different biopsies of normal, and to internal negative controls. The analysis was performed by using the Leica DMRA fluorescence microscope, with PL FLUOTAR 25 x / 0.75 OIL, and PLAN APO 40 x / 1.25 – 0.75 OIL objectives, and with a DAPI (UV) excitation fluorescence filter, and a FITC (blue) excitation fluorescence filter (Leica Microsystems B.V., Rijswijk, The Netherlands). Expression of the different factors was evaluated by means of previously described semi-quantitative methodologies\(^\text{30}\) with slight modifications. Staining intensity was scored as category 0 (negative to weak), 1 (medium), and 2 (strong). Staining grade was scored as 0 (0–20%), 1+ (21-60%), 2+ (61-100%) according to the percentages of positively stained cells as determined semi-quantitatively. Using intensity and percentage of positive cells, tumors were classified as being negative in case of the staining intensity was categorized as 0 with the number of positive cells graded as 0. Tumors were classified as positive, in case at least the staining intensity was categorized as 1 and the percentage of positive cells was at least 1+. Cases that did not fit into these two categories were classified as intermediate.
Cytometric Bead Array (CBA) Multiplex Assays

Biopsies from both normal and tumor tissues were used to obtain lysates by dissolving them in Greenberger Lysis Buffer (GLB). Briefly, the procedure was as follows: tissues were collected in liquid nitrogen and subsequently frozen down and suspended in GLB (150mM NaCl, 15mM Tris 1mM MgCl₂, 1% Triton X-100, Protease Inhibitor) diluted 1:1 with PBS. Biopsies were homogenised on ice and then placed on ice for 30 minutes. The disrupted tissues were spun down at 4000 rpm for 7 minutes, and the supernatant was collected and spun down at 1400 rpm for 10 minutes. One aliquot was taken to measure protein with the BCA protein assay kit (Pierce chemical co. Rockford, IL, USA). Lysates were used to perform Cytometric Bead Array (CBA) using the CBA Inflammation kit (BD) following the manufacturer’s instructions as previously described.1 Acquired data were analyzed using BD CBD calibration and analysis software.

BCA Protein assay

To determine the protein concentration in the analyzed samples, the bicinchoninic acid (BCA) protein assay kit was used. Briefly, 5μl of the lysates obtained as described above were used and incubated in a 96 wells plate together with protein assay reagents A and B following the manufacturer’s instructions, and the protein content was measured spectrophotometrically using Plate Manager® software.

Statistical analysis

Statistical analyses were performed using the software Graph Pad Prism®. Statistical tests were applied to 17 independent experiments. Differences between the values were determined using both Students’ paired t-test, one way ANOVA and Mann-Whitney test, as appropriate. Significance was determined as p<0.05.

RESULTS

Patient's characteristics and routine histology

The study was approved by the Academic Medical Center (Amsterdam, The Netherlands) Hospital’s Medical Ethical Committee. All 17 patients enrolled in the study obtained informed consent and gave written permission. The population constituted of 15 males and 2 females, the mean age was 74 (range 50-87), and cancers were at variable stages of differentiation. Histopathology of the H&E staining of the biopsies as evaluated by an expert pathologist showed EAC in all the 17 patients (Fig.3A). Patient data are summarized in table 1.
Quantitative PCR (Taqman) for IL-8 and IL-6

The levels of IL-8 in the tumor tissues as compared to normal tissues, by QPCR were as follows: IL-8 was not detectable in any of control (normal) tissues (relative expression mean=0.93±0.06SEM), with one exception. In contrast, 58% of the tumor samples showed an up-regulation in the level of IL-8 (Relative expression mean=1751±1170SEM); it is worthy to note that the levels between patients were extremely variable (Fig.1). IL-6 expression using Taqman revealed that these tumors are not characterized by any expression of this particular cytokine neither in normal controls nor in the tumor tissues.

Cytometric Bead Array for IL-8, IL-1β and IL-6

CBA data were comparable to results as obtained by QPCR for IL-8 and IL-6 expression. In this investigation, we included as well the analysis for IL-1β. At protein levels, normal mucosa tissues samples didn’t show any level of IL-8, IL-1β, IL-6 with the exception of 2 out of 17 (11%) samples with a mean of 10pg/ml±3.2SEM, 29±20.4SEM and 0.1±0.1SEM, respectively. In contrast, in tumor tissue samples, 80% of the patients showed a significant up-regulation of IL-8 as compared to the matched normal mucosal controls with a mean expression of 145pg/ml±73.3SEM (Mann-Whitney test, p<0.05). Yet it was noted that the levels between patients are highly variable (Fig. 2A). IL-6 in the tumor samples was detected only in 4 out of 17 patients (23%): the differences between IL-6 as measured in the normal tissues was not significant as compared to the tumor tissues (Mann-Whitney test, p=0.13, Fig. 2B). IL-1β was detected in 86% of the tumor tissues, and appeared to be significantly up-regulated in the tumors as compared to normal tissues (mean=29pg/ml±20.4SEM), with a mean of 420pg/ml±276SEM (Mann Whitney test, p<0.05, Fig.2C).
Immunohistochemical analysis for VEGF, TGF-β, COX-2, IDO, CXCL3 and CXCR1

IHC of normal mucosa was negative for VEGF, TGF-β, and COX-2 in 15 out of 17 cases, or only detectable in limited areas of the normal tissues, particularly in the inflammatory areas and germinal centres, with a weak intensity of the staining (classified negative) (Fig.3B). In the cancer mucosa, expression of COX-2 protein was found in all tumor samples. All tumors were classified as positive for COX-2 and showed expression in 61% to 100% of cells (grade 2+) in combination with strong staining intensity (category 2). COX-2 expression was mainly seen on the membrane of epithelial cells, but also partly on stromal cells (i.e., fibroblasts and endothelial cells). VEGF was classified as positive in 87% of the tumor samples which expressed as well high percentages of positive cells (Grade 1+) and intermediate intensity staining (category 1). VEGF staining was particularly confined to the cytoplasm and at membrane level in the epithelial cells layer, but often patchy and localized especially in the germinal areas. TGF-β was classified as positive in 80% of the tumors, mostly showing strong intensity (category 2) in a high percentage of cells (grade 1+), and predominantly in epithelial cells. IDO was positive in all of the tumor cases, and a strong expression (category 2) with a grade of 1+ staining was especially seen in the stromal cells, which possibly are infiltrates, close to the epithelial cells. CXCL3 was positive in 87% of the tumors, staining was mainly confined to the epithelial cells, particularly in the germinal centres, with a grade of 1+ of strong staining intensity (category 2). Its receptor, CXCR1, was positive in 93% of the tumor samples, and

Figure 2: CBA analysis of protein levels in EAC patients specimens. A: IL-8 was found to be significantly higher expressed in tumor samples as compared to normal samples (Mann Whitney test, p<0.05). B: IL-6 was not detected in any sample, with the exception of 2 samples (out of 17) that did not show any significant difference when comparing normal and tumor samples (Mann Whitney test, p=0.2). C: IL-1β level was significantly higher in tumor samples as compared to normal samples (Mann-Whitney test, p<0.05).
was specifically expressed in the epithelial cells, with a grade of 1+ and intermediate positivity (category 1) (Fig.3C). We found that for all these factors, there was a significant up-regulation in the tumor tissues, as compared to normal tissues.

Figure 3. A) H&E of the normal and tumor samples. B) IHC performed on frozen normal and tumor tissues sections for (A) IDO, (B) VEGF, (C)TGF-β, (D)CXCL3, (E)CXCR1 and (F)COX-2. Positive staining of all these factors is seen in the EAC biopsies as compared to normal biopsies (G-M, Fig.3C).
DISCUSSION

Tumor resistance to immunological attack is thought to be a result of alteration in the production of specific factors, for instance co-stimulatory and co-inhibitory molecules, or an imbalanced activation of effector T-cells and regulatory T-cells. Such an essential balance is especially altered in patients with advanced stage cancers. These malignancies for instance present high levels of inflammatory molecules, cytokines, chemokines, and tumor infiltrating immunosuppressive cells. Only few factors had been systematically studied in the immunological microenvironment of EAC. It has been broadly demonstrated that factors, such as cyclin D1, EGFR, Her-2/Neu, APC, TGF-beta, P53, Bcl-2, NF-kB, COX-2, E-cadherin, β-catelin, are highly expressed at RNA and protein levels, and correlate with poor prognosis and tumor progression in several cancers, including EAC.

Our study was aimed at gaining insight in the EAC microenvironment to study the balance of a panel of immunomodulating substances and factors, which characterize these cancers. Our results show that EAC features a particular pattern of immune-inhibiting factors. Significant high levels of VEGF were seen in 90% of the patient’s samples. Such a high expression was as well previously observed in EAC. Several studies have confirmed that VEGF is known to enhance and allow the process of metastasis. This factor has been previously described in EAC as a molecule, which plays a role in lymphatic metastasis via lymphangiogenesis and angiogenesis, indicating that pharmacological inhibition of VEGF might be useful to hinder progression of these cancers. VEGF is further described as an inhibitor of DC differentiation and maturation. For instance, in vitro data have shown that anti-VEGF neutralizing antibodies block the negative effects of tumor cell supernatants on DC maturation. These results are even more interesting when looking at the simultaneous expression with COX-2 in the tumors. We observed that COX-2 is highly expressed by the tumor epithelial cells and stroma in significant levels as compared to normal tissues. Similar results were seen in previous studies, where it was shown that the expression of COX-2 and VEGF correlated with metastatic stage, and may be used as important prognostic factors. As COX-2 inhibition is clinically very feasible and has limited side effects, our results may be interpreted that such inhibition is an attractive adjuvant strategy in EAC. Another key player in the mechanism of tumor immune-escape is TGF-β. Many tumors, including EAC, overexpress TGF-β, and high circulating levels of TGFβ1 in cancer patients are frequently associated with poor prognosis. Von Radhen et al, showed that high expression of TGF-β, is an important promoter of tumor growth in a series of primary resected esophageal adenocarcinomas. It has been previously shown that TGF-β mediates hypo-responsiveness of the memory T cells in human lung.
tumor microenvironment in situ by blocking TCR signalling\textsuperscript{52}. Furthermore, adoptive transfer of tumor-reactive, TGF-β-insensitive CD8+ T cells into immunocompetent mice was able to eradicate lung metastasis of mouse prostate cancer\textsuperscript{53}. In addition, there is evidence that TGF-β in general reduces T-cell functions\textsuperscript{54}. In our study, we could detect positive or intermediate positivity for TGF-β in the majority of the population, confirming the results of previous studies\textsuperscript{55}, suggesting that this factor may be of critical importance in mediating immune-evasion is EAC and that inhibition of its signaling might be beneficial in patient treatment.

Next to these factors, we selected molecules which are thought to be important in the process of tumor immune-escape, but which expression has never been investigated in EAC. These include the enzyme IDO, the chemokine CXCL3 and its receptor CXCR1. Several studies in the last few years have been supporting the idea about the pivotal role of IDO in the inhibition of T-cells\textsuperscript{50,56-58}. In vitro and in vivo, and in different models, this enzyme, responsible for the process of tryptophan catabolism, has attracted the interest of immunologists because of its potent inhibitory effect on T-cells. It has been observed that tryptophan depletion consequently induces apoptosis of T-cells. IDO and its enzymatic products have been found in many tumors, and, as proved by functional studies, T-cell responses can be greatly enhanced by inhibiting its action. Therefore, it was of utmost importance in our study to investigate whether or not this factor is expressed in esophageal cancers. Our results show that this molecule is highly up-regulated in the stroma of EAC tumor tissues, as shown by IHC, and we therefore speculate that IDO as a potent inhibitor of the immune-defence is one of the active mechanisms in these cancers leading to immune-escape. Another important factor is the CXC ligand CXCL3, a chemokine found previously to be up-regulated in EAC patients biopsies by using Serial Analysis of Gene Expression (SAGE) in our laboratory (data not shown). It has been demonstrated that CXCR receptors and related ligands, are profoundly associated to angiogenesis and tumorigenesis\textsuperscript{13,59}. Our results show that this molecule is extensively overexpressed in EAC, especially with regard to epithelial cells. This pattern correlates with the expression of the receptor CXCR1, which was also found to be extremely high expressed in tumor tissues as compared to normal tissues. Surprisingly, the levels of IL-8, another important factor known to interact with VEGF and therefore, also responsible for metastatic behaviour of cancers\textsuperscript{59-60}, was found to be significantly up-regulated in tumors both at RNA and protein level\textsuperscript{61-64}. Significantly high levels of IL-8 were detectable in human melanoma cell lines, and it has been demonstrated that reduction of the protein level of human IL-8 in human melanoma cell lines is associated with a substantial inhibition of their colony formation and proliferative rate\textsuperscript{14}. High secretion of IL-8 has as well been described in human colon carcinoma cell lines and in colon carcinoma\textsuperscript{65}. This may confirm the hypothesis that there
is an important cross-talk between these two factors, which can be responsible for the aggressiveness of EAC. Based on previous studies, and analysing these results, we may speculate that, as shown for other cancers, targeting either IL-8 or VEGF and inhibiting this intense cross-talk, can be an attractive strategy to decrease tumor immune-escape in EAC patients.

Analyzing the results for other cytokines, such as IL-1β, we could observe that the level of this pro-inflammatory cytokine was significantly increased in the tumor tissues. These results confirm a previous study where it was shown that expression of IL-8 and IL-1β are significantly up-regulated not only in EAC, but as well in its precursor lesions, Barrett’s esophagus and even in esophagitis. The high levels observed in the tumor tissue samples, confirmed what previously has been reported on the IL-1β role in cancer progression and its correlation with poor prognosis. This can be explained as a role of this molecule in maintaining an inflammation status in cancer tissues, promoting non-favourable impaired interactions between the different cell populations that characterize the tumor tissue.

As expected, IFN-γ levels in lysates of tumor biopsies did not show any significant difference as compared to the normal tissues, indicating that effector T-cell responses are not initiated in these cancers, or have been inhibited. We observed a significant correlation between the results as obtained for the cytokines at RNA levels and those as measured at protein level. Our results are similar to a previous study where low levels of IFN-γ were detected in patients with EAC.

Surprisingly, the levels of IL-6 observed in the tumor tissues as compared to normal, both at RNA and protein levels, did not show a significant difference, indicating that probably this well known immuno-suppressive factors, in this particular cancer does not exert a main role in terms of decreasing patient immune-responses. This confirms previous observations, which have reported on the role of IL-6 in the chronic inflammation status in gastro-esophageal cancers.

In summary, in this study we have analyzed the esophageal cancer tumor microenvironment, in order to define the balance of factors that characterize its immuno-surroundings. We selected several factors known to be involved in the mechanism of tumor immune-escape in order to establish the pattern of expression of these factors both at RNA and protein level. Here we describe a specific tumor micro-environment, in which significant up-regulation of particular immuno-suppressive factors are observed, while several important immunogenic factors are lacking. We hypothesize that these molecules are involved in the inhibition of an appropriate and efficient immune-response, which lead to escape from immune surveillance. These factors may potentially contribute to resistance to DCs vaccination and other immunotherapeutic approaches. Targeting these molecules in the future may lead to significant improvement of the efficacy of immunotherapies.
REFERENCES


Chapter 5

Trastuzumab synergistically acts with HER-2 specific CTL to induce tumor cell lysis in Esophageal Adenocarcinoma cell lines

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ABSTRACT

Esophageal adenocarcinoma (EAC) is a highly aggressive disease that with the conventional treatment strategies has extremely poor prognosis. In a previous study we demonstrated that CTL mediated DC therapy is a promising novel treatment option for EAC. EACs are known to have high amplification of the HER-2 gene, and therefore it is of interest to explore combinatorial treatment strategies employing DC immunotherapy and anti-HER-2 treatment for this patient group. Previous studies showed that HER-2-derived peptides are naturally processed as Cytotoxic T-cell (CTL) epitopes that can be recognized by tumor-specific CTLs. The humanized antibody against HER-2, Trastuzumab, has potent inhibitory effects on HER-2 overexpressing tumors. In addition, this antibody may induce an increase of HER-2 epitope presentation via MHC-Class I molecule on tumor cells that may mediate a HER-2 specific CTL response and tumor cell lysis. In this study we first, determined the HER-2 gene amplification and protein expression in EAC cell lines, and investigated the biological activity of Trastuzumab. Secondly, we ex-vivo evaluated whether Trastuzumab and HER-2-specific CTLs act synergistically for inducing tumor lysis, and then did the same using PBMCs of patients with and without HER-2 overexpressing EAC. The HLA-A2/HER-2 positive cell lines, OE33 and OE19, and HLA-A2 positive/HER-2 negative cell line Bic-1 were used to test the effect of Trastuzumab performing an MTT assay. In the same time, HLA-A2 matched donor DCs were loaded with HER-2 RNA to obtain HER-2 specific CTLs. Hereupon, a cytotoxicity assay was performed, which revealed a synergistic effect of Trastuzumab and HER-2 specific CTLs in lysing the HER-2 overexpressing EAC cell line OE33, but not the HER-2 negative Bic-1. Most interestingly, PBMCs of HLA-A2 matched EAC patients with high HER-2 amplification induced significantly higher levels of lysis of the HER-2 positive cell lines as compared to PBMCs of patients without HER-2 amplification. This effect was further enhanced in case cells were pretreated with Trastuzumab. These results show that Trastuzumab treatment of HER-2 expressing cell lines enhances the susceptibility to lysis by HER-2-specific CTLs. Furthermore, we provide evidence that EAC patients with HER-2 overexpressing cancers might have circulating anti HER-2 CTLs that upon treatment with Trastuzumab may lead to an improvement for a specific anti HER-2 cytolytic immune response.
INTRODUCTION

HER-2/neu is a 185 KDa transmembrane glycoprotein with tyrosine kinase activity and extensive homology to the epidermal growth factors receptor⁴.

HER-2 is ubiquitously expressed in a large proportion of epithelial cancers, and it has been widely reported to be overexpressed, mostly via gene amplification, in several aggressive cancers², such as in 25-30% of ovarian and breast cancer³, 35-45% of pancreatic carcinoma⁵, in a wide range of colorectal carcinomas⁶, and in 30-80% of esophageal adenocarcinoma⁷-¹⁴. Since cytotoxic T-lymphocytes (CTL) response for several HER-2 peptides have been observed in cancer patients, the HER-2/neu protein appears to be immunogenic, and therefore is an ideal Tumor Associated Antigen that can be employed for anti-cancer immunotherapy¹⁵-¹⁹. The fully humanized antibody Trastuzumab (Herceptin), which specifically targets the extracellular domain of the HER-2 protein, exhibits potent growth inhibitory activity against HER-2 overexpressing tumors²⁰-²³. Trastuzumab has been used in clinical studies showing encouraging results, particularly when used as therapy for breast cancer²⁴-²⁶. Applying Trastuzumab as an additional treatment option could be an attractive strategy to consider for tumors with a notoriously poor prognosis such as EAC. This cancer, in Western countries, is the major type of esophageal cancer; the incidence is the most rapidly increasing compared to other malignancies and has exceeded 5 per 100,000 per year²⁷,²⁸. Conventional treatment options for these patients can guarantee only low survival rate: the main treatment of EAC is surgical resection, yet, even after curative resection with or without neo-adjuvant or adjuvant chemo- and or radiotherapy, the median survival is less than 2 years²⁹,³⁰. In addition, several studies suggested that HER-2 amplification/overexpression may be relevant for these tumor entities¹⁰. In an ex-vivo study, we previously showed that DC vaccines could be an advantageous approach for curing esophageal cancer⁴⁴. For these reason, we questioned whether immunotherapy of EAC in combination with the use of Trastuzumab could be a valid approach as a more effective strategy to treat EAC patients. The underlying mechanisms of the action of Trastuzumab include blockade of the signalling pathway as well as induction of apoptosis in tumor cells and inhibition of tumor cell growth through down-regulation of the HER-2 receptor and the enhancement of the immune system response such as Antibody Dependent Cellular Cytotoxicity (ADCC)³¹,³². It has been previously shown that HER-2 overexpressing cell lines treated with Trastuzumab are more susceptible to killing by HER-2 specific CTLs³³,³⁴. To date, only one report describes the possibility of applying Trastuzumab as a treatment for EAC patients showing an overall toxicity, which was not increased as compared to previous studies³⁵.
In this study, we examined the level of gene amplification and protein overexpression in diverse EAC cell lines by Fluorescence In Situ Hybridization (FISH) and immunocytochemistry (ICC). We first evaluated the effect of Trastuzumab against HER-2 expressing EAC cell lines and showed that Trastuzumab induces cell growth arrest in HER-2 overexpressing cell lines. We then evaluated whether Trastuzumab increases the capacity of HER-2 specific CTLs to induce lysis of tumor cells and showed that in fact pre-treatment with Trastuzumab can enhance CTLs lytic capacity for the HER-2 positive cell line OE33, but not for the HER-2 negative cell line Bic-1. In addition, we showed that PBMCs isolated from patients with HER-2 amplification induce lysis of Trastuzumab pre-treated EAC cell lines, while this effect is significantly less when employing PBMCs of patients without HER-2 amplification.

MATERIALS AND METHODS

Cell Lines and PBMCs from EAC patients.
EAC cell lines OE19 and OE33 were obtained from ECACC (Porton Down, Wiltshire, SP4 DJG, UK), Bic-1 was a gift from Dr. N.S. Buttar (Mayo Clinic, Rochester, MN, USA). OE19 and OE33 were maintained in RPMI 1640 medium with addition of L-glutamine, antibiotics and Fetal Calf Serum (FCS). Bic-1 was kept in DMEM medium with addition of L-glutamine, antibiotics and FCS. All the cell lines were incubated at 37ºC and 5% CO₂ and medium was refreshed every three days.

Peripheral blood mononuclear cells (PBMCs) were separated from blood obtained from healthy donors and EAC patients by density gradient centrifugation using Ficoll-Paque (Amersham, Pharmacia, Piscataway, NJ, USA) and cryo-preserved in a DMSO/FCS (2:8) solution (Merck, Darmstadt, Germany; GIBCO BRL, Grand Island, NY, USA, respectively) for further use.

Fluorescent in situ hybridization (FISH)
EAC cell lines OE33, OE19 and Bic-1 were trypsinized at a confluence of 70-80%, spun down and collected in PBS. A Cytospin (Shandon Cytospin 4 Cytocentrifuge, Thermo, Waltham, MA) was used to generate a single layer of cells on a glass slide as described before. The cytospin slides were dried overnight at RT, and then stored at -80ºC until FISH analysis was performed. We used directly labeled fluorescent chromosomal centromeric probes (CEP) for chromosome 17 and 17q11.2- q12 (Her2/neu), obtained from Vysis (Downers Grove, IL). DNA-FISH was performed according to the manufacturer’s instructions provided by Vysis as described before by our group. After the FISH procedure, 100 interphase nuclei of tumor cells were scored per slide by an experienced scorer (A.M Rygiel).
using Olympus BX61 fluorescent microscope (Germany). The categories of Her-2/neu gene abnormalities were determined by calculating the ratio of Her-2/neu locus signals (orange) to chromosome 17 centromere signals (green). The following categories were distinguished: ratio <2 were considered as having no amplification, ratios \( \geq 2 \) and <5 were considered as low amplification and ratio \( \geq 5 \) was considered as high amplification. More than two green signals (CEP 17) accompanied by the same number of orange signals (Her2/neu locus) was considered to be indicative of polysomy of chromosome 17 (ratio 1:1). Following these criteria the cases were classified as displaying a polysomy of chromosome 17 (cutoff \( \geq 3 \% \) of abnormal nuclei) and an amplification of Her2/neu locus (cutoff \( \geq 5 \% \) of abnormal nuclei) as established before.

**Fluorescent immuno-cytochemistry of cultured cells.**

Cultured cells were plated in a 24 well plate at a concentration of \( 1 \times 10^4 \)/ml and grown on coverslips. After 24 hours, cultured cells were fixed directly into the well plate for 20 minutes in Phosphate Buffered Saline (PBS) with 4% Paraformaldehyde (PFA) and 0.1% Triton X-100 and then washed in PBS. Blocking of aspecific antigens was performed by incubating slides for 45 minutes with PBS containing 1% BSA and 10% Fetal Calf Serum (FCS). Slides were washed with PBS and incubated overnight at 4°C with the appropriately diluted primary HER-2 antibody, c-erbB-2/HER-2/neuAb-17 (Lab Vision Corporation, Freemont, CA, USA) in PBS with 1% BSA and 0.1% Triton X-100. After washing with PBS, cells were incubated with a mouse anti-human secondary FITC conjugated antibody (Dako, Denmark) 1:500 diluted in PBS. Cells attached on coverslips were placed on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides and mounted with DAPI (Roche, Mannheim, Germany)/vectashield (Vector laboratories Inc, Burlingame, CA, USA) 1:1000.

**Flow cytometric analysis for HLA-A2 typing.**

To assess the HLA-A2 type of the EAC cell lines, cells were harvested, washed and re-suspended in a concentration of \( 1 \times 10^6 \) in FACS buffer (5 g BSA, 0.1 g NaN3, 100 mM EDTA in 1 L PBS), then incubated with anti-human HLA-A2 antibody (BD) PE conjugated. Cells were stained for 30 minutes on ice in the dark, then spun down and re-suspended in FACS buffer, and analyzed with the FACSCALIBUR apparatus (Becton-Dickinson, Franklin Lakes, NJ, USA). Analysis of the result was done using Cell Quest Pro Software (BD). Isotypes antibodies were used as control.

**Isolation of monocytes and lymphocytes from peripheral blood**

Human monocytes were isolated from peripheral blood of healthy volunteers HLA-A2 positive using the Ficoll-Percoll gradient separation method,
previously described by Milano et al.\textsuperscript{46}. Once monocytes were separated from lymphocytes, both populations were re-suspended in IMDM and washed twice. Monocytes and lymphocytes were counted and either used immediately or cryo-preserved in a DMSO/FCS (2:8) solution (Merck, Darmstadt, Germany; GIBCO BRL, Grand Island, NY, USA, respectively). A further separation of monocytes from lymphocytes was done using a Percoll (Amersham Biosciences Europe Freiburg, Germany) gradient separation, following manufacture’s protocol. Briefly, PBMCs were washed in RPMI 1640 medium (BioWhittaker-Cambrex Bioscience, Walkersville, MD, USA) at 1500 rpm for 5 minutes twice. At the mean time, Percoll was mixed with 10X Phosphate Buffer Saline (PBS) to obtain Standard Isotone Percoll solution (SIP), and then with IMDM medium (BioWhittaker), to obtain three solutions at different concentration (60, 47.5 and 34% SIP). PBMCs were then re-suspended in 2.5 ml 60% SIP, then 5 ml 47.5% SIP and finally 2 ml 34% SIP were added to the 60% SIP cell suspension and a centrifugation at 3100 rpm for 45 minutes was performed. Once monocytes were separated from lymphocytes, both populations were re-suspended in IMDM and washed twice. Monocytes and lymphocytes were counted and either used immediately or cryo-preserved in a DMSO/FCS (2:8) solution (Merck, Darmstadt, Germany; GIBCO BRL, Grand Island, NY, USA, respectively).

**Generation of HER-2 mRNA by in vitro transcription.**
HER-2 mRNA was made as previously described\textsuperscript{36}. Briefly, to generate HER-2 mRNA, plasmid pSPJC1 with an insert coding for HER-2 (a gift from Prof. A.Ulrich, Martinsried, Germany) was used. This plasmid allows in vitro transcription under the control of an SP6 promoter. The plasmid was linearized using Ndel (MBI Fermentas, St. Leon-Rot, Germany) and in vitro transcription was subsequently performed using Ambion in vitro transcription kit with an SP6 promoter (Ambion, Nieuwerkerk a/d, IJssel, The Netherlands), and the manufacture’s procedure was followed. The in vitro transcribed mRNA was purified using RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacture’s instructions. The RNA was then dissolved in RNase-free H$_2$O and stored at -80 °C until required. Spectrophotometry was performed with 1μl of total RNA to quantitate on the Nanodrop® (Nanodrop Tecnologies, type ND-1000, Wilmington, USA).

**Electroporation of monocytes with HER-2 mRNA**
Monocytes characterized as CD14+, CD83-, CD86-, CD209- were used for electroporation with HER-2 mRNA. The electroporation procedure was performed as previously described by F.Milano et al.\textsuperscript{46}, using Amaza cell lines Nucleofector procedure, kit V. After electroporation, 1x10$^5$ monocytes were
plated in 24 well plates with 1 ml of Cell Gro Medium (Cellgenix, Freiburg, Germany) with the addition of 2% of Penicillin/Streptomycin (GIBCO, Breda, The Netherlands), and 800U/ml IL-4 and 1000U/ml GM-CSF for obtaining immature DCs. After six days, IL-1β, TNF-α and LPS (10 ng/ml, 25 ng/ml, 0.02 ng/ml respectively) were added to obtain mature DCs. The electroporation efficacy of HER-2 mRNA was assessed through measuring HER-2 expression by FACS analysis. Cells were harvested and spun down at 1500 rpm for 10 minutes, then re-suspended in FACS buffer in a concentration of 1x10⁶ cells/ml and sorted by the FACS apparatus for direct measurement of HER-2 extra cellular domain using anti-human HER-2 primary monoclonal antibody anti HER-2 clone 9G6.10 (Lab Vision, Fremont, CA). Expression was measured in monocytes 24 hours after electroporation (day one) and daily on eight subsequent days after electroporation, during the maturation of the monocytes into mature DCs.

**Induction of HER-2 specific CTLs**
After eight days from the isolation of monocytes, the obtained HER-2 loaded mature DCs were twice co-incubated for seven days with autologous lymphocytes in a proportion of 1:4 (5x10⁵ DCs and 2x10⁶ lymphocytes) in 24 wells plates in Cell Gro medium with 2% penicillin-streptomycin (GIBCO). As control population, lymphocytes were as well incubated with DCs which underwent the electroporation procedure but without adding HER-2 RNA.

**Mixed Leucocyte Reaction (MLR) using [³H] thymidine incorporation assay**
The capacity of DCs to present antigens and evoke a lymphocytes response was tested in an autologous Mixed Leucocyte Reaction (MLR). Briefly, mature DCs electroporated either with HER-2 RNA and DCs electroporated with no antigen source were co-cultured in flat-bottom 96-well plates (Nunc, Roskilde, Denmark) with lymphocytes in IMDM medium at different target-effector ratios, starting from 4x10⁵/well, down to 1.25x10² DCs (APC) per well, while each well contained a fixed number of 5x10⁴ lymphocytes. After 5 days in culture, 0.2 μCi [³H] thymidine (Amersham Biosciences, Amersham, UK) was added to each well and the incorporation of radioactivity was measured after 16 hours using liquid scintillation counting.

**MTT assay of Trastuzumab treated cells**
To evaluate the effect of Trastuzumab on EAC cell lines, cultured cells were treated with increasing concentration of Trastuzumab, namely 2.5, 5 and 10 μg/ml, and left untreated for control purposes. Cells were harvested after 24 hours, 48 hours and 72 hours of treatment with Trastuzumab, transferred on an enzymatic 96 wells plate and co-incubated with MTT[3-(4,5-dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide, for 4 hours. Ethanol absolute (96%) was added to stop the reaction, and results were read on a multi-well scanning spectrophotometer (ELISA reader).

**Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) assay.**

In this assay the Trastuzumab treated EAC cell lines were incubated with HLA matched HER-2 specific CTLs or lymphocytes obtained by stimulation with DCs which underwent the electroporation procedure, but without the addition of HER-2, and cell lysis was measured. The HLA-A2 positive cell lines OE33 and OE19 that were as well HER-2 positive, and the HLA-A2 positive Bic-1 cell line that was HER-2 negative was incubated for 48 hours with 10μg/ml Trastuzumab. Hereupon, 1x10^4 treated and not treated cells (target) were co-incubated with effector cells, i.e., the HER-2 specific CTLs or non specific CTLs, and PBMCs isolated from patients with or without HER-2 amplification, at various E:T ratios, namely 5:1, 2.5:1, 1.25:1 and 0.62:1, in 100 μl of RPMI 1640 in a 96-well plates in triplicate for 4 hours at 37°C. The percentage of cytotoxicity was measured by the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega, Madison, USA). This is a colorimetric cytotoxicity assay, which quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatant is measured with a 30-minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour will be proportional to the number of lysed cells. Wavelength absorbance data were collected using a multi-well scanning spectrophotometer (ELISA reader). The percentage of specific cytotoxicity was calculated according to the formula: % specific lysis = [(Experimental-Effector Spontaneous-Target Spontaneous)/(Target Maximum-Target Spontaneous)] x100.

**Cytometric Bead Array**

Supernatant from the different samples was collected after performing the cytotoxicity assay and used for measuring IFN-γ release from activated HER-2 specific CTLs. The procedure was followed as described previously, following the manufacture’s instruction, and samples were examined in triplo for each condition.

**Apoptosis in esophageal adenocarcinoma cell lines**

Each cell line (1x 10^4 cells) was cultured in 2 ml of RPMI 1640 and DMEM as appropriate with or without Trastuzumab (10 μg/mL) at 37°C for 48 hours in a six-wells plate. After incubation, apoptosis in each cell line was measured by staining with APC-conjugated Annexin-V and 7aad via probe as previously described and a staining procedure was followed as described before.
Statistics
To evaluate statistical differences between groups, Student’s t test was used. Statistically significant difference was considered at p< 0.05.

RESULTS

The status of HER-2 expression and gene amplification in the OE33, OE19 and Bic-1 EAC cell lines and of the EAC patients.
HER-2 status was determined in OE33, OE19 and Bic-1 EAC cell lines at DNA level, performing FISH. The analysis revealed that OE33 and OE19 are characterized by a high HER-2 amplification as compared to Bic-1 that only showed polysomy of the chromosome 17 (Fig.1A, Table1). The same procedure was used for determining HER-2 status in EAC patient brushes, and revealed high HER-2 amplification in one patient and no amplification in two patients. To validate the results obtained by FISH, we analyzed EAC cell lines at protein levels by using ICC. Upon analysis it was possible to observe a strong overexpression of HER-2 in OE33, a weaker expression in OE19, whereas Bic-1 showed no staining (Fig.1B).

Figure 1: HER-2 status as measured in EAC cell lines. HER-2 status analysis in three different EAC cell lines show that, at DNA level, OE33 and OE19 are characterized by high HER-2 gene amplification, whereas Bic-1 exhibits polysomy of chromosome 17. At protein level, OE33 and OE19 both overexpress HER-2, although OE19 has a lower percentage of positive cells, as compared to controls. Bic-1 cell line does not overexpress HER-2 (Fig.1 and Table 1).
Table 1: HER-2 STATUS

<table>
<thead>
<tr>
<th>CELL LINES</th>
<th>FISH</th>
<th>ICC</th>
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</thead>
<tbody>
<tr>
<td>OE33</td>
<td>High amplification</td>
<td>+++ positivity</td>
</tr>
<tr>
<td>OE19</td>
<td>High amplification</td>
<td>++ positivity</td>
</tr>
<tr>
<td>Bic-1</td>
<td>polysomy</td>
<td>negative</td>
</tr>
</tbody>
</table>

Trastuzumab inhibits growth, but does not increase apoptosis, in HER-2 overexpressing EAC cell lines.

To evaluate the potential inhibitory activity of Trastuzumab on HER-2 overexpressing cell lines, namely OE33 and OE19, cells treated at different time points and different concentrations of Trastuzumab were analyzed by MTT, and, further, stained by Annexin V and 7aad to assess the level of apoptosis comparing treated with not treated cells. Growth arrest in not treated cells was on average 0.21±0.02SEM. Inhibition of growth induced by Trastuzumab in OE33 at 24 hours using 2.5μg dose, was 0.1±0.01SEM (Paired T-test, p<0.05). Increment of the Trastuzumab dosage at this time point did not further influence cell viability. After 48 hours, growth inhibition was not significantly different as compared to not treated cells (One way ANOVA, Bonferroni Post-Test, p=0.7). After 72 hours, using 2.5μg dose, cell viability significantly decreased to 0.2±0.02SEM, Paired T-test, p<0.05). Further increasing the doses of Trastuzumab at this time point did not result in enhanced sensitivity of the OE33 cell line (One way ANOVA, Bonferroni Post Test, p=0.05, Fig.2A). The basal cell viability before treatment of OE19 was on average 0.34±0.02SEM. After incubating the cells with a dose of 2.5μg of Trastuzumab for 24 hours OE19 cell viability significantly decreased as compared to not treated cells (0.2±0.03SEM, Paired T-test, p<0.05). Significant inhibition was observed as well using a 5μg and 10μg dose (Mean 0.2±0.01SEM and 0.18±0.02SEM, respectively, paired T-test, p<0.05). After 48 hours of treatment, using a 2.5μg dose showed significant growth inhibition as compared to the not treated cells (0.2±0.03, Paired T-test, p<0.05). Using 5μg dose induced no further decrease in cell viability: 0.2±0.01 (Paired T-test, p=0.08), but 10μg dose again induced a significant decrease in cell viability as compared to not treated cells (Paired T-test, p<0.05, Fig.2B). After 72 hours of treatment, either using 2.5, 5, or 10μg dose, inhibition of growth was not significantly different as compared to not treated cells (One way ANOVA, Bonferroni Post Test, p=0.7). In Bic-1 (EAC cell line HER-2 negative, used as control), no differences were observed at different time point using different concentrations of Trastuzumab (Fig.2C).

Annexin V and 7aad staining as performed to assess the apoptosis level induced in EAC cell lines by the treatment with Trastuzumab showed no significant increase of apoptosis levels in the Trastuzumab treated cell lines as compared to not treated cell lines, either expressing or not expressing HER-2 (Fig.2D).
HER-2-Specific CTLs.

FACS analysis performed using the HER-2 specific antibody, confirmed that, after 24 hours from the electroporation procedure, HER-2 was successfully expressed by the HER-2 RNA loaded DCs, and, at day eight of the maturation process the mature DCs were still able to present HER-2 epitopes, as demonstrated by significantly

Figure 2: The biological activity of Trastuzumab against EAC cell lines

A) EAC HER-2 positive cell lines treated with increasing concentration of Trastuzumab, and at different time points, showed progressively decreased cell viability in both OE33 and OE19, whereas Bic-1 (HER-2 negative EAC cell line) taken as control, showed no sensitivity to Trastuzumab.

B) Apoptosis levels as measured in EAC cell lines treated for 48 hours with 10μg of Trastuzumab did not show any increase in the apoptosis level, with no differences between treated and not treated cells.
higher MFI of 1608±448SEM as compared to the control Isotype antibody and not electroporated cells (MFI=167±70 and 112±38, respectively, Paired T-test, p<0.01, Fig. 3A). In the MLR, lymphocytes, which were stimulated by HER-2 electroporated monocytes showed a significantly higher proliferation rate (Mean 2409±809SEM) as compared to the lymphocytes stimulated by monocytes electroporated without any antigen source (control) (Mean 702±131SEM, Paired T-test, p<0.05). The obtained CTLs were subsequently used for the cytotoxicity assays.

**Trastuzumab enhances HER-2 specific CTL lysis of OE33 but not of OE19 or Bic-1.**

The cytolytic effects of the different lymphocyte populations on the Trastuzumab treated and not treated EAC cell lines were studied. The overall percentage of lysis of the not treated HER-2 overexpressing EAC cell lines OE33 and OE19 after incubation with the HER-2 specific CTLs was 18.6%±10.2SEM, while the effect obtained with the (control) aspecific CTLs was 1±0.7SEM. After pre-treating the cell lines with Trastuzumab the lysis of the OE33 cell line further increased to 32%±13.8SEM, while the effect induced by aspecific CTLs was 2.4±2.4SEM (Fig.4A). This additional lytic effect of Trastuzumab pre-treatment was not visible for the OE19 cell line, which showed a mean cytotoxicity level of 2.2±1.1SEM in all the different conditions (Fig.4B). As expected, Bic-1, which does not overexpress HER-2, did not show any sensitivity to the anti HER2 specific CTLs with or without Trastuzumab pre-treatment (Mean cytotoxicity in all the samples=12±1.5SEM, Fig.4C).
PBMCs of EAC patients with HER-2 gene amplification enhance Trastuzumab ADCC against HER-2–expressing EAC cell lines.

The ADCC assay was performed using PBMCs of HLA-A2 positive EAC patients with or without HER-2 positive cancers, against OE33, OE19, and Bic-1. The ADCC showed that PBMCs isolated from EAC patients with HER-2 amplification induced significantly higher cytolytic effects on HER-2 overexpressing EAC cell lines, and was 4.5±0.8SEM as compared to controls (CTLs), which was 0.5±0.5. Cytotoxicity further increased to 11.6±2.8SEM when cells were pre-treated with Trastuzumab (Fig.5). No cytolytic effect was observed in case PBMCs isolated from patient without HER-2 amplification were employed.

IFN-γ production by CTLs

After four hours of incubation, the supernatant obtained from the co-culture of CTLs and the EAC cell line OE33 either treated or not treated with Trastuzumab was collected and used to measure the IFN-γ release by CTLs. Production of IFN-γ was significantly higher when CTLs were co-incubated with OE33 as compared to the basal IFN-γ levels: 75.5pg/ml ±20.5SEM versus 5.4pg/ml±3SEM, T-test,
Chapter 5

p<0.05. The release was even higher in case CTLs were incubated with OE33 pre-treated with Trastuzumab: 96.5pg/ml±11.5 versus 5.4pg/ml±3SEM, T-test, p<0.05 (Fig.6). These results corresponded with the lysis activity as measured in the cytotoxicity assays.

**DISCUSSION**

There are many mechanisms linked to the therapeutic effect of Trastuzumab, including the blockade of signaling pathways, inhibition of tumor cell growth\textsuperscript{20}, activation of apoptotic signals of tumor cells\textsuperscript{37} and enhancement of ADCC\textsuperscript{20,31}. In previous studies, Mimura K. et al. have demonstrated that Trastuzumab is able to induce ADCC against HER-2–expressing esophageal Squamous Cell Carcinoma (SCC). This effect can be explained by the fact that Trastuzumab induces the rapid loss of both cell surface and total cellular HER-2. This antibody-induced down-regulation of HER-2 is a result of accelerated endocytosis and correlates with the tumor-inhibitory potential of the antibody\textsuperscript{20,36}. In our study, we examined the HER-2 status of several EAC cell lines and showed that OE33 and OE19 are characterized by high level of HER-2 gene amplification, as previously shown\textsuperscript{13}, whereas Bic-1 is characterized by only polysomy of chromosome 17. These results were confirmed at protein levels by ICC, which clearly showed a high protein overexpression in OE33 and OE19, as compared to controls and the Bic-1 EAC cell line. This explains the growth inhibitory effect observed in the two former cell lines, particularly visible already after 24 hours of treatment, whereas Bic-1 growth was not affected by the treatment with Trastuzumab. We further observed that the anti-proliferative activity of Trastuzumab for HER-2 expressing EAC cell lines does not correspond to a higher level of apoptosis in OE33.
treated cells as compared to not treated cells, confirming prior observations. One important mechanism that has been shown previously, is that the tumor-inhibitory antibodies to HER-2 enhance ubiquitination of HER-2. Protein tagged to ubiquitin is known to be targeted to the proteasome, which cleaves the protein into small peptides. These peptides are then transported into the endoplasmic reticulum, loaded onto MHC class I molecules, and transferred to the cell surface for recognizing CTLs. In addition, some recent studies demonstrated that HER-2-specific CTLs, generated from an HLA-A2 positive patient with HER-2 positive breast cancer, lysed the HER-2 positive SKOV3tA2 breast cancer cells in an HLA-A2 restricted manner. These results suggest that Trastuzumab sensitized HER-2-overexpressing tumors inducing lysis in a MHC class I-restricted manner by HER-2-specific CTLs. Based on these prior findings, we investigated whether this phenomenon can as well be observed in EAC cell lines using HER-2 specific CTLs. Our findings confirmed that treatment with Trastuzumab significantly increased the capacity of HER-2 specific CTLs to lyse the HER-2 overexpressing EAC cell line OE33. Surprisingly, this effect was not observed in OE19 EAC cell line, although this cell line does overexpress HER-2, but at a lower level (Fig 1B). The reason for this might be that the OE19 cell line has an impairment in the MHC-Class I antigen presentation machinery, which is a subject for our future studies. Reasonably, this effect was not observed in Bic-1 cell line, which does not overexpress HER-2. Interestingly, performing the ADCC test using lymphocytes not specific for HER-2, no differences were observed in OE33 cell line, as well as in OE19 and Bic-1, providing evidence that in this particular circumstance, the lytic effect is rather mediated by anti HER-2 specific CTLs than by NK cells. Kono et al. have shown both in gastric cancer and in esophageal squamous cell carcinoma that treatment with Trastuzumab induces ADCC using PBMCs from healthy donors, while PBMCs from patients showed a significantly lower ADCC, meaning that probably these patients may have impaired NK T-cell functions. In our study, we first selected patients with advanced stage disease with or without HER-2 gene amplification and compared the level of ADCC in these two different patient populations. The Trastuzumab-mediated ADCC of PBMCs from EAC patients with HER-2 amplification was significantly higher as compared to that observed in patients who did not present HER-2 gene amplification. The result partly confirms the observations of Kono et al. In addition we show that there is an important difference in ADCC between PBMCs obtained from EAC patients with HER-2 gene amplification compared to those without HER-2 gene amplification. It is likely that patient with high HER-2 levels are already sensitized and have developed HER-2 specific CTLs, of which the lysis capacity may be enhanced by the action of Trastuzumab. This confirms other studies where it was shown that some breast cancer patients with HER-2 positive tumors have pre-existent T- and B-cell-mediated immunity to the HER-2 protein. These results have been
later confirmed by the same groups and by others\textsuperscript{41,42}. It is important to note that cancer patients exhibiting natural antibody and T-cell immunity to HER-2 do not develop autoimmune responses, suggesting that HER-2 specific antibodies and T cells generated because of HER-2 overexpression do not recognize basal HER-2 expression on normal epithelial cells\textsuperscript{41}. Our findings described here, confirming that Trastuzumab enhances the cytolytic activity of HER-2 specific CTLs and showing that this antibody can mediate a significantly higher ADCC in HER-2 positive EAC patients, encourages us to initiate combination therapies including both the antibody and CTLs directed against HER-2. In summary, this study shows that the EAC cell line OE33 with high HER-2 amplification is susceptible to Trastuzumab treatment. In addition, we found that specific anti HER-2 CTL responses can be enhanced through pre-treatment with Trastuzumab. We further provide data that EAC patients with HER-2 expressing cancers may have specific anti HER-2 CTLs, and that these patients may highly benefit from the combinatorial therapy by combining Trastuzumab with anti HER-2 CTL mediated immunotherapies.
REFERENCES


Chapter 6

Bone Morphogenetic Protein (BMP)-4-expressed in esophagitis induces a columnar phenotype in esophageal squamous cells

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ABSTRACT

BACKGROUND & AIMS: Barrett Esophagus (BE) is a metaplastic condition in which normal squamous esophageal epithelium is replaced by columnar epithelium. It is proposed that one of the possible mechanisms is dedifferentiation of squamous epithelium into columnar epithelium. The pathophysiology through which this metaplasia occurs is unknown. A recent study by SAGE analysis showed that Bone Morphogenetic Protein 4 (BMP-4) is uniquely expressed in BE. In this study the role of the BMP pathway in the metaplastic transformation of normal squamous cells into columnar cells was examined. METHODS: Tissues from esophagitis-BE patients and in an esophagitis-BE rat model were examined for the activation of the BMP-pathway. Short term cultures of primary normal squamous esophageal cells were treated with BMP-4, and cell biological changes were examined by Western blot analysis, immuno-histochemistry and microarrays. RESULTS: In both human and rat tissues the BMP pathway proved to be activated in esophagitis and BE. Upon incubation of squamous cell cultures with BMP-4, the cytokeratin (CK) expression pattern showed a shift which was consistent with columnar epithelium. Involvement of the BMP pathway was suggested by up-regulation of Phosphorylated-Smad 1/5/8 (P-Smad 1/5/8) that was effectively blocked by Noggin, a BMP antagonist. Comparison of the gene expression profiles of squamous cells, BMP-4 treated squamous cells and BE cells, showed a significant shift in the profile of the BMP-4 treated squamous cells towards that of the cultured BE cells. CONCLUSIONS: These results suggest that the BMP pathway could play a role in the transformation of normal esophageal squamous cells into columnar cells.
INTRODUCTION

Barrett Esophagus (BE) is a pre-malignant condition of the distal esophagus, which is associated with an increased risk of developing esophageal adenocarcinoma. Esophageal adenocarcinoma is one of the most rapidly increasing, lethal cancers in Western countries and is becoming a major health concern.1-3 BE is believed to be a metaplastic change in which the normal stratified squamous epithelium in the distal esophagus is replaced by columnar epithelium.4 This process is assumed to be the result of longstanding gastroesophageal reflux disease (GERD). GERD is a common condition that can be found in 20-30% of the general Western population.3 It has been estimated that BE can be found in 6-12% of patients who suffer from GERD and it is assumed that individuals with GERD and esophagitis subsequently may develop BE.6,7 Although the concept of metaplasia is largely accepted, the actual pathophysiology by which these changes take place is unknown. In order to identify genes specifically involved in the transition of normal squamous esophageal mucosa into metaplastic BE, we previously used serial analysis of gene expression (SAGE), a technique to compare the expression profile of BE with that of its surrounding epithelia, i.e. normal squamous esophageal and gastric cardia mucosa. BMP-4 was found to be abundantly and uniquely expressed in the SAGE library of BE, but not in normal squamous or cardia epithelium. BMP-4 is a protein belonging to the transforming growth factor beta (TGF-β) family. Members of the TGF-β family are involved in controlling cellular differentiation, migration and proliferation.8 BMPs are 30-35 kD hetero- or homodimeric proteins. They were originally shown to play a role in bone formation, but were also found to be essential during embryonic development.9,10 BMPs induce the formation of a heterodimeric complex of the BMP receptor type I and type II. This receptor complex signals downstream by phosphorylating specific BMP receptor regulated Smads (Smad 1, 5 and 8). The P-Smad 1/5/8 forms a heterocomplex with Smad 4, this complex translocates into the nucleus, where certain target genes, such as ID2, can be transcribed.11 Previous studies demonstrated that BMPs are induced during inflammation and injury.12-15

In this study, we provide evidence that reflux-induced metaplastic transformation of inflamed squamous esophageal mucosa to columnar type mucosa is mediated by BMP-4. Consequently, manipulation of this pathway could potentially prevent the development of BE and thus reduce the occurrence of esophageal adenocarcinoma.

MATERIALS AND METHODS

Patient and Biopsy Specimens
Biopsy specimens obtained during routine surveillance endoscopies from 28 non-dysplastic BE patients were used. The median age was 67 years (range 39-88
years) and 13 patients were male. The median length of the BE segment measured endoscopically was 5.4 cm (range 2-13 cm). All patients were on long term proton pump inhibitor therapy. Paired biopsies were obtained from the Barrett segment as well as of normal squamous esophagus. The Barrett biopsies were taken at least 2 cm above the gastroesophageal junction. Normal squamous epithelium was taken at least 2 cm above the Barrett segment. Special care was taken to avoid sampling Barrett areas containing islands of quamous mucosa. Additional biopsies from 6 patients, who had at least had grade B (Los Angeles classification) esophagitis were collected. Four of these patients were male and the mean age was 54 years (range 27-70 years). Paired biopsies were taken next to each other from the inflamed squamous mucosa and normal squamous segment of these patients. None of the esophagitis patients had a concomitant BE. The inflammation was histologically confirmed on the pairwise taken control biopsies.

**Primary Cell Culture**

Biopsies specimens of 15 BE patients were used for tissue culture. Biopsies were collected during endoscopic procedure and immediately placed in MCDB 153 medium (Sigma Chemical Co., St Louis, MO, USA) on ice. Biopsy specimens were processed within 4 hours after endoscopy. The explant method described by K. Washington et al. was used. Primary cell cultures were initiated by maintaining the cells in Barrett plus media, as previously described. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C and the media was replaced twice weekly. After two to three weeks of culturing, squamous cells were incubated with 100 ng/ml recombinant h BMP-4 (R&D Systems, Minneapolis, MN, USA) for several time points and/or 50 µg/ml recombinant Mouse noggin/Fc chimera (R&D) for several time points. For the preparation of cells on glass slides, cells were harvested and re-suspended in medium, dropped on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides and air-dried overnight.

**RNA isolation**

Total RNA for Microarray was isolated from squamous, BMP-4 treated squamous, and Barrett primary cell cultures derived from three different patients, using TRIzol Reagent (according to manufacturer’s instructions, Life Technologies Inc, Invitrogen, Breda, The Netherlands). Upon isolation, spectrophotometry was performed with 1 µl of total RNA to quantitate on the Nanodrop® (type ND-1000). Assessment of the quality was performed with the RNA 2100 Pico Labchip kit (Agilent Technologies, Amstelveen, The Netherlands) using 1 µl of total RNA.

**Microarray analysis: RNA Amplification, Labeling and Hybridization**

Microarray analysis was performed for three different patients. The mRNA was double amplified using the Amino Allyl MessageAmp kit (Ambion, Austin,
USA). The labeling, hybridization and data extraction were performed at ServiceXS (Leiden, The Netherlands). Briefly, 20 ng total RNA was mixed with 1 µl of T7 Oligo(dT) primer in a total volume of 12 µl. Primer and template were denatured by incubating at 70°C for 10 minutes and annealed by putting the reaction tubes on ice. The First Strand Reaction was performed by adding 8 µl Reverse Transcription Master Mix (containing 10x First Strand buffer, Ribonuclease Inhibitor, dNTP Mix and Reverse Transcriptase) and incubating at 42°C for 2 hours. Second Strand cDNA Synthesis was done by adding 63 µl Nuclease-Free Water, 10 µl 10x Second Strand Buffer, 4µl dNTP Mix, 2µl DNA polymerase and 1 µl RNase H and incubating at 16°C for 2 hours. cDNA purification was done according to the manufacturer’s protocol (Ambion). *In vitro* transcription was initiated by addition of 3 µl UTP solution (50mM), 12 µl ATP, CTP, GTP Mix (25mM), 4 µl T7 10x Reaction Buffer and 4 µl T7 Enzyme Mix and incubated at 37°C for 5 hours. Purification of aRNA was done according to manufacturers protocol (Ambion). The second round First Strand Synthesis was done using 2 µg purified aRNA, adding 2 µl 10x First Strand Buffer, 1 µl Ribonuclease Inhibitor, 4 µl dNTP Mix and 1 µl Reverse Transcriptase and incubating at 42°C for 2 hours. After adding 1 µl of RNase H, samples were incubated at 37°C for 30 minutes. Five µl of T7 Oligo (dT) primer was added and the sample was denatured by incubating at 70°C for 10 minutes and annealed by putting the samples on ice. The second round Second Strand cDNA Synthesis was done by adding 58 µl Nuclease-Free Water, 10 µl 10x Second Strand Buffer, 4 µl dNTP Mix and 2 µl DNA polymerase and incubating at 16°C for 2 hours. cDNA purification was done according manufacturers protocol (Ambion). *In vitro* transcription was initiated by addition of 2 µl aaUTP Solution (50mM), 12 µl ATP, CTP, GTP mix (25mM), 3 µl UTP Solution (50mM), 4 µl T7 10x Reaction Buffer and 4 µl T7 Enzyme Mix and incubation at 37°C for 9 hours. Qiagen’s RNeasy mini spin columns (Qiagen, Benelux B.V., Venlo, The Netherlands) were used for purification of the cRNA as described in Agilent’s user manual. Dye Coupling Reaction was performed using 5 µg amino allyl aRNA, 9 µl Coupling Buffer and 11 µl NHS ester dye, prepared according manufacturers protocol (Amersham, Buckinghamshire, United Kingdom). After an incubation at room temperature for 30 minutes, 4.5 µl 4M Hydroxylamine was added and incubated at room temperature for 15 minutes. Dye labeled aRNA was purified according to the manufacturers protocol (Ambion) and the samples were checked on concentration and dye incorporation on the Nanodrop ND-1000. The cRNA yield was between 91 µg and 123 µg. Hybridization was performed with 600 ng or 1 µg of each labeled target together with control targets, fragmentation and hybridization buffer at 60°C for 17 hours onto Agilent Human Whole Genome Oligo arrays (Amsterdam, the Netherlands) following the manufacturer’s protocol.
Microarray Imaging, Data and Statistical Analysis

The microarray slides were washed following the instructions in the user manual and scanned on the Agilent dual laser DNA microarray scanner. The microarray data was normalized using the Agilent feature extraction software (version 7.5) to correct for the background correction and analyzed using the Rosetta Resolver v5.0 Expression Data Analysis System (Rosetta BioSoftware, Seattle, USA).

To compare the normal squamous cells with the BMP-4 incubated squamous cells we hybridized every sample to the BE sample and as such used the BE cells as the standard. Statistical analysis was done using the Chi square test to test whether there was a significant difference in genes that were more than 2 fold up- or down-regulated in the normal squamous cells compared to the BE cells versus the BMP-4 treated squamous cells compared to BE cells. To make a more strict statistical comparison between the non treated squamous cells and BMP-4 treated squamous cells, with the expression levels of the BE cells as the reference, we build new ratios using Ratio-split software of the Rosetta Resolver Package. Using this Ratio-split, statistical analysis, ANOVA (on intensity data) was performed for the 3 different experiments (p<0.01).

Western blot analysis

The squamous cells treated with BMP-4 were washed with ice cold PBS and lysed with 200 µl lysis buffer (Cell Signaling), 1 mM Pefablock (Sigma). Biopsy material from squamous, esophagitis and BE specimens were collected in liquid nitrogen and immediately placed at -80°C. Samples were subsequently lysed with 200 µl lysis buffer 1 mM Pefablock for western blot analysis. The lysates were sonicated and then centrifuged at 20g for 10 minutes at 4°C. The pellet were discarded and the protein concentration was measured with the BCA protein assay kit (Pierce chemical co. Rockford, IL, USA). Lysates were diluted 1:2 in protein sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercapto ethanol; 20% glycerol; 1 mg bromphenol blue) and incubated for 5 minutes at 95°C. Twenty µg of protein per lane was loaded onto SDS-PAGE and subsequently transferred onto PVDF membrane (Millipore, Amsterdam, The Netherlands). The blots were blocked with 2% BSA in Tris Buffered Saline supplemented with 0.1% Tween-20 (TBST) for one hour at room temperature and washed in TBST before overnight incubation at 4°C with primary antibody in 2% BSA in TBST. Blots were then washed with TBST and incubated for 1 hour at room temperature in 1:1000 Horse Radish Peroxidase (HRP) conjugated secondary antibody in 2% BSA in TBST. After a final wash with TBST, blots were incubated for 5 minutes in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Fuji LAS3000 illuminator (Fuji Film Medical Systems, Stamford, USA). The antibodies used and dilutions are summarized in Table 1.
Table 1: Antibodies as used for Western blotting and immuno-histochemistry.

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Rat model of BE

Six-week-old male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN, USA). A detailed description of the procedure and conditions of the preparation of the BE rat model has been described by Buttar et al. Briefly, 8 rats underwent a midline laparotomy and Levrat’s esophagojejunostomy to induce enteroesophageal reflux. Four rats were kept under the same conditions but with sham procedure, for control purposes. Rats were euthanized between 20 to 22 weeks post-operatively using CO₂ narcosis, intramuscular injection of 12 mg/kg xylazine hydrochloride and removal of 5 ml intra-cardiac blood. The whole body was cooled to 4°C and a midline incision was made from the laryngopharynx to the lower abdomen. The site of anastomosis was identified by finding the polypropylene sutures. It was freed of any adhesions and then dissected free of surrounding tissue up to the laryngopharynx. The esophagus was then cut at the level of the larynx and 2 mm above the site of anastomosis and opened longitudinally. Finally esophageal tissues from 4 control rats and 4 rats that had both esophagitis and BE were collected. Tissue samples from inflamed esophagus and columnar lined esophagus were divided in two equal parts. Half of the tissue was formalin fixed and sectioned to confirm the diagnosis of esophagitis and BE, the remaining half was stored at -80°C until processing for immunohistochemistry and H&E staining. The animal study was approved by the animal care committee (IACUC) at the Mayo Clinic (Rochester, MN, USA).

Fluorescent immuno-histochemistry of tissues and cultured cells

Biopsies from squamous mucosa, esophagitis and BE specimens were embedded in Tissue-Tek® Optimum Cutting Temperature compound (Sakura Finetek, USA, Torrance, Calif.) at -20°C and sectioned using a cryo-microtome (Microm HM 550) in serial cryostat sections of 6 μm, placed on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides and air-dried overnight. For routine histological examination, of each block one slide was stained by hematoxylin and eosin. Cultured cells were harvested and dropped on Superfrost + (Menzel
Glaser, Braunschweig, Germany) glass slides and air-dried overnight. Slide preparations were fixed for 20 minutes in Phosphate Buffered Saline (PBS) with 4% Paraformaldehyde (PFA) and 0.1% Triton-X 100 and then washed in PBS. Blocking of a-specific antigens was performed by incubating slides for 45 minutes with PBS containing 1% BSA and 10% Fetal Calf Serum (FCS). Slides were washed with PBS and incubated overnight at 4°C with the appropriately diluted primary antibody in PBS with 1% BSA and 0.1% Triton-X 100. After incubation the slides were washed with PBS and incubated with the secondary FITC conjugated antibody (Dako, Denmark) 1:500 diluted in PBS. Slides were washed and mounted with DAPI (Roche, Mannheim, Germany)/vectashield (Vector laboratories Inc, Burlingame, CA, USA) 1:1000. The antibodies used and dilutions are summarized in Table 1.

Confocal microscopy on rat esophagitis-BE animal model
Rat tissues were embedded in Tissue-Tek® Optimum Cutting Temperature compound (Sakura Finetek, USA, Torrance, Calif.) at -20°C and sectioned using a cryo-microtome (Microm HM 550) in serial cryostat sections of 6 μm, placed on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides and air-dried overnight. For routine histological examination, of each block one slide was stained by hematoxylin and eosin. Other slides were used to perform fluorescent immunohistochemistry for BMP-4, Smad 1/5/8 and ID2, as described above and examined by confocal microscopy using a Leica TCS-SP2 filter-free Spectral Confocal Microscope (Heidelberg GmbH, Molecular Imaging Center, Bergen, Norway). A 40X magnifying objective was used with the numerical aperture (NA) at 1.25, type HCX PL fluotar.

RESULTS

Expression of BMP-4 and downstream targets in BE, squamous and inflamed squamous epithelium of patients.
In a recently published SAGE analysis we found that BMP-4 is 19 fold up-regulated in BE compared to normal squamous epithelium.\(^8\) Validation by Western blot analysis of BE and normal squamous esophagus biopsies confirmed the SAGE data and showed a high expression of BMP-4 on protein level in BE and a low expression in normal squamous esophagus. Importantly, we now found a high expression of BMP-4 in the inflamed squamous epithelium (Figure 1). Western blot analysis demonstrated that Smad4 as well as BMP receptors: BMP RIA and BMP RII, were expressed in BE, in normal squamous epithelium and in esophagitis (Figure 1), yet ID2, a downstream BMP-4 target, and P-Smad 1/5/8 were only detectable in BE and in esophagitis but not in normal non-inflamed squamous epithelium (Figure 1). Immunohistochemistry of biopsy specimens demonstrated the expression of BMP-4, P-Smad1/5/8 and ID2 in esophagitis and BE while there was no expression in normal squamous epithelium.
Figure 1: Expression of proteins of the BMP pathway in BE, esophagitis and normal squamous epithelium. A: western blot analysis of BMP-4, P-Smad 1/5/8, ID2, BMP RIA, BMP RII and Smad4 expression in BE and normal squamous esophagus biopsies and B: in inflamed squamous (esophagitis) and normal squamous epithelium. Results show that BMP-4 and its downstream targets P-Smad 1/5/8 and ID2 are expressed in BE and inflamed squamous epithelium, while they are not expressed in normal squamous epithelium. Smad4 and both receptors, BMP Receptor IA and BMP Receptor II are expressed in BE inflamed squamous epithelium as well as normal squamous epithelium. Actin was used as a control.

Figure 2: BMP-4 pathway expression in normal squamous esophagus, inflamed squamous epithelium and BE of patient biopsy specimens. Hematoxylin and eosin (H&E) stainings and immuno-histochemistry with FITC (green) conjugated antibodies for BMP-4, P-Smad 1/5/8 and ID2 on biopsy specimens of normal squamous epithelium, esophagitis and BE is shown. DAPI (blue) was used as a nuclear counter stain. IHC shows that normal keratinizing esophageal mucosa does not show any staining for BMP-4, P-Smad and ID2. The inflamed squamous epithelium and Barrett mucosa show an increased expression of BMP-4 and nuclear expression of the downstream targets including P-Smad 1/5/8, and nuclear and cytoplasmic staining for ID2, confirming activation of the BMP pathway. A 100 and 40 magnifying objectives were used.
BMP-4 expression in the inflamed esophagus and BE was typically localized in the stromal tissue, while P-Smad 1/5/8 was localized in nuclei suggesting transcriptional activity (Figure 2). ID2, which is a transcription product of P-Smad, is seen in the nuclei and cytoplasm of the epithelial cells (Figure 2).

**BMP-4 and downstream targets expression in BE, squamous and inflamed squamous epithelium in the BE rat model.**

Normal keratinizing squamous epithelium is found in the control rats (Figure 3). Reflux esophagitis and intestinal type of metaplasia of the mucosa, resembling BE, are found in the rats above and at the esophago-jejunostomy site (Figure 3). Confocal analysis of immunohistochemical stainings of the fresh frozen material of the normal rat esophagus showed no expression of BMP-4, P-Smad 1/5/8 and ID2. BMP-4 and the downstream BMP-4 targets, P-Smad 1/5/8 and ID2, were only seen in the inflamed esophagus and in the BE resembling epithelium (Figure 3). The pattern and localization of the expression of these factors were comparable to the patterns found in the human biopsy specimens as shown in Figure 2.

![Figure 3: BMP-4 pathway expression in normal squamous esophagus, inflamed squamous epithelium and of the rat-BE model.](image)

The Hematoxylin and eosin (H&E) stainings and immuno-histochemistry (IHC) using FITC (green) conjugated antibodies for BMP-4, P-Smad 1/5/8 and ID2 of the tissue from the rat esophagus-BE model is shown. The left column shows normal non-keratinizing squamous epithelium, inflamed squamous mucosa and columnar mucosa resembling BE. IHC shows keratinizing normal rat esophageal mucosa with negative staining for BMP-4, and negative staining for the BMP-4 downstream targets. The inflamed squamous epithelium and columnar epithelium show activation of the BMP-4 pathway with increased expression of BMP-4 and nuclear expression of the downstream targets P-Smad 1/5/8 and ID2. DAPI (blue) was used as a nuclear counter stain. A 40X magnifying objective was used.
**BMP-4 activation in primary cultures of normal squamous esophageal cells**

Time-course incubation of primary cultured normal squamous cells with 100 ng/ml recombinant BMP-4 was performed. Western blot analysis showed that after 5 minutes of BMP-4 incubation there are increased levels of P-Smad 1/5/8 (Figure 4A). These phosphorylation levels were increased even more at 10 minutes and 20 minutes of incubation.

The untreated squamous cell cultures did not show any phosphorylation of Smad 1/5/8 (Figure 4A). Pretreatment of squamous cells with the BMP-4 antagonist Noggin for 10 minutes decreased the phosphorylation level of Smad 1/5/8 to a basal level, indicating that the BMP pathway was blocked (Figure 4B).

**Figure 4: P-Smad 1/5/8 expression in cell cultures stimulated with BMP-4.** A: Western blot analysis of P-Smad 1/5/8 in primary cultured Barrett and squamous esophageal cells. In cultured squamous cells there is no phosphorylation of Smad 1/5/8, while cultured Barrett cells show high levels of P-Smad 1/5/8. Upon treatment of the squamous cell cultures with BMP-4 for 5, 10 or 20 minutes P-Smad 1/5/8 levels are up-regulated. B: This up-regulation in phosphorylation level of Smad 1/5/8 is effectively inhibited when the squamous cells are incubated with Noggin (a BMP antagonist). Actin was used as a control.

**Cytokeratin expression in cultured squamous cells before and after BMP-4 treatment**

CK10 and CK13 are normally only expressed by squamous cells, while CK7 and CK20 are more specific for columnar types of cells such as BE. After 5 hours of treatment of squamous cells with BMP-4, Western Blot analysis showed up-regulation of expression of CK7 and CK20, while these cells were still expressing CK10/13 (Figure 5). Immuno-histochemistry showed that the normal squamous cells do express CK10/13 but not CK7 or CK20, while the cultured Barrett cells express CK7 and CK20 but not CK10/13 (Figure 6). After 5 days of treatment of squamous epithelial cells with BMP-4 the up-regulation of CK7 and CK20 becomes clearly visible by immunohistochemistry. At this time point decreased expression of CK10/13 was also seen. (Figure 6).
Chapter 6

Whole human genome microarray analysis

The gene expression profiles from tissues of three different patients of BMP-4 treated, no BMP-4 treated squamous cells as well as Barrett cell cultures were obtained and compared. The overall gene expression profile of the BMP-4 treated squamous cells in comparison to the Barrett cells and untreated squamous cells, indicate that there was a shift of the gene expression profile of the BMP-4 treated squamous cells towards the expression level of the cultured BE cells. Analysis of

Figure 5: Expression of Cytokeratin 7, 10/13 and 20 in cultured squamous cells incubated with BMP-4.

Western blot analysis of CK7, CK10/13 and CK20 expression in cultured squamous cells and Barrett cells shows that in control (not treated) squamous cell cultures there is high expression of CK10/13 but CK7 or CK20 expression are absent. After 5 hours of incubation of the squamous cultures with BMP-4, CK7 and CK20 are up-regulated, while CK10/13 is still present. Actin was used as control.

Figure 6: Cytokeratin 7, 10/13 and 20 expression in cultured Barrett, non treated squamous and BMP-4 treated squamous cells. Immunohistochemistry with FITC (green) conjugated antibodies shows the expression of CK7, CK10/13 and CK20 in primary cultured Barrett, squamous esophageal cells, and BMP-4 treated squamous cells. DAPI (blue) was used as a counter stain. Normal squamous cells show no expression of CK7 and CK20 but strongly express CK10/13 (left row) while Barrett cells are positive for CK7 and CK20 but not for CK10/13 (right row). After 5 days of treatment of the cultured squamous cells with BMP-4 CK7 and CK20 are up-regulated, while CK10/13 expression is decreased (middle row). In the middle row (squamous cells treated with BMP-4) sub-panels are included showing in detail the cytokeratin pattern change in single cells.
Figure 7: Microarray analysis comparing differentially expressed genes in squamous and BMP-4 treated squamous cells versus Barrett cells. A: Scatter Plots showing the comparison of microarray analysis of Barrett versus non treated squamous cells, and B: Barrett versus BMP-4 treated squamous cells. C: Two dimensional dendrogram created from hierarchical clustering, according to the Pearson correlation, with the genes in a cluster displayed below each average. The length of each branch of the tree is proportional to the distance between the averages. In the upper panel, depicted in red are genes which are up-regulated in the cultured squamous cells, depicted in green are genes which are down regulated in squamous cells. In the lower panel, depicted in red are the genes which are up-regulated in the BMP-4 treated squamous cells and in green the down regulated genes. The expression of BE is taken as the reference (black).
a subset of genes that are at least 2-fold up-or down-regulated showed that there were on an average 11099 genes differentially expressed when comparing no BMP treated squamous versus Barrett cells, while on average 8226 differentially expressed genes were found when comparing BMP-4 treated squamous versus BE cells. The decreased number of the at least 2-fold differentially expressed genes as seen upon treatment of the squamous cells with BMP-4 was statistically significant (Chi square test; p=0.0001). More strict statistical analysis of the 3 different microarray experiments showed that comparing the expression levels in the BE cells with non treated squamous cells revealed that 446 genes were significantly differently expressed, while the comparison of the BE cells with the BMP-4 treated squamous cells showed that 392 genes were significantly differentially expressed, which was a decrease of 12% (ANOVA; p<0.01). Hierarchical clustering of the significantly differentially expressed genes, using Pearson’s correlation, showed that upon treatment of the squamous cells with BMP-4, there was a shift towards the expression pattern which resembled BE cells. This is depicted in figure 7C that shows that genes highly expressed in the squamous cells (red in the upper panel) decrease in level of expression after BMP-4 treatment (green in lower panel), while a set of genes which are not expressed in the squamous cells (green in the upper panel) gain expression in the BMP-4 treated squamous cells (red/black, lower panel). Note that most of the induced genes in the BMP-4 treated cells have a similar level (black) compared to BE cells.

DISCUSSION

In previous studies BMPs have been found to directly change the pathophysiology of certain inflammatory conditions. Due to their chemotactic activity on inflammatory cells and fibroblasts, BMPs have been considered to influence inflammatory processes in adults. BMP-4 is a protein belonging to the TGF-β protein family. So far, the involvement of members of the TGF-β family in BE has not been investigated in detail. With microarray analysis Barrett et al. found that a TGF-β superfamily protein was one of the genes that was up-regulated in BE. With the use of SAGE, we recently found that BMP-4 is exclusively expressed in BE and absent in normal squamous epithelium. Since BE is associated with chronic inflammation as a result of reflux of gastric contents damaging the esophageal mucosa, we hypothesized that these inflammatory changes could induce the production of BMP-4, which subsequently triggers trans-differentiation of squamous epithelial cells into a columnar cell type, resembling metaplastic Barrett mucosal cells, that replaces the normal squamous mucosa.
In this study, we provide evidence which supports that BMP-4 might have an important role in the process of transformation of squamous epithelium towards a mucosa, which resembles columnar metaplasia and that this process is initiated in the inflamed squamous mucosa. Using Western blot and immunohistochemistry we found that BMP-4 and its downstream targets, P-Smad 1/5/8 and ID2 are present in patient biopsies from BE and squamous epithelium in the area of esophagitis, but not in normal non inflamed squamous esophageal mucosa (Figure 1 and 2). This indicates that indeed in inflamed esophageal mucosa, BMP-4 is up-regulated and its downstream pathway is activated. The finding of BMP receptors (RIA and RII) expression in normal squamous esophageal tissue supports the hypothesis that under certain conditions the BMP pathway can be activated in squamous epithelium. Analysis of the tissues obtained from a rat model in which reflux esophagitis and subsequently BE is induced through chronic reflux of gastro-duodenal contents into the esophagus, confirmed that the BMP pathway is activated in both the inflamed esophageal mucosa and BE. This was illustrated by an increased expression of BMP-4 and the downstream targets P-Smad and ID2 (Figure 3). We further investigated in an ex-vivo model whether the BMP pathway was active in BE mucosa and whether BMP-4 could induce dedifferentiation of cultured squamous cells into a columnar cell type that resembles BE. To this end we established short term primary cultures of normal squamous epithelial cells and Barrett cells from patient biopsies. Treatment of squamous epithelial cells with recombinant human BMP-4 for 5, 10 or 20 minutes showed expression of P-Smad 1/5/8, that increased in time, while the control squamous cell cultures did not show any expression level of activated Smad proteins (Figure 4A). Blocking the pathway by pre-incubating squamous cells with the BMP antagonist Noggin before treatment with BMP-4, showed a decreased phosphorylation level of Smad 1/5/8 (Figure 4B). From these ex-vivo experiments we conclude that the BMP pathway can be activated in normal squamous cell cultures and that this effect can be inhibited by Noggin.

To further study the trans-differentiation process of the squamous epithelium into columnar metaplasia, we examined the CK expression profiles in BMP stimulated and non stimulated squamous cells. The CK expression is known to be specific depending on type, location and differentiation of the epithelium and is therefore important for characterizing certain epithelia, for instance characterizing the difference between BE and normal squamous cells.8, 23-26 CK7 and CK20 are known to be expressed in BE and other columnar types of mucosa but not in normal squamous mucosa. In contrast CK10/13 is expressed in normal squamous mucosa but not in BE or other columnar types of mucosa.8, 27, 28 After 5 hours of incubation of squamous cell cultures with BMP-4, Western blot analysis revealed that there was up-regulation of CK7 and CK20, while CK10/13 was still present in these cells (Figure 5). After 5 days treatment of squamous epithelium with BMP-
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4, CK7 and CK20 induction could be demonstrated by immunohistochemistry, whereas non-treated squamous cells did not express these CKs. At this point we noted a decreased CK10/13 expression in the BMP-4 treated cells (Figure 6). From these results we can conclude that treatment with BMP-4 induces a shift in the CK expression pattern towards a CK expression pattern that resembles that of BE. To further investigate to what extent a columnar type of mucosa is induced by BMP-4, whole human genome arrays were used to compare the total gene expression profiles of primary BE cell cultures with the BMP-4 treated and non treated squamous cell cultures. A remarkable change of the gene expression profile was seen of the BMP-4 treated squamous cell cultures (Figure 7). When taking into account those genes that were more abundantly (> 2-fold) up- or down-regulated, the overall number of differentially expressed genes diminished by 26%. Yet, using multivariate analysis for the three experiments and only take into account those genes that were significantly differentially expressed between the squamous cultured cells and the BMP-4 treated squamous cells, with respect to expression levels in the BE cells, we observed a reduction of 12%. To further demonstrate that BMP-4 down regulates a set of squamous specific genes and induces expression of a set of genes related to columnar metaplasia, we performed hierarchical clustering using Pearson’s correlation. From this analysis we could conclude that after BMP treatment of the squamous cells, within the significantly differentially expressed genes, there is a set of ‘squamous’ specific genes that is significantly reduced expressed while the expression of a set of ‘novel’ genes is induced. Most of the induced genes have expression levels that are similar to expression levels in the Barrett cells, but few genes have a higher expression than what is seen in the BE cells (Figure 7C). The latter phenomenon may be explained by the experimental conditions in which the level of BMP stimulation may be beyond physiological levels and counteracting effects and feed back mechanisms that normally may be present in the in vivo situation were lacking.

In summary, we made a novel observation that BMP-4 plays an potentially important role in the process of metaplasia as occurs in the distal esophagus in which normal squamous epithelial cells are dedifferentiated into columnar type of cells. We demonstrated that the BMP pathway is activated in inflamed esophageal squamous epithelium to an extent, which is similar to what was seen in the columnar epithelium of both human and rat tissues and that the BMP-4 pathway can be blocked effectively with Noggin, a specific BMP antagonist. In our ex-vivo experiments the dedifferentiation of normal squamous cells towards a columnar cell type as induced by BMP-4, which was particularly illustrated by changes of the CK expression patterns. This was also illustrated by a shift in the gene expression profile of the BMP-4 treated squamous cells that towards that of Barrett epithelial cells. Our findings suggest that BMP-4 is involved in initiating the process of transformation of normal squamous esophageal mucosa into a
columnar type of cells. Once we better understand the mechanisms involved in the process of de-differentiation initiated by BMP-4, manipulation of the BMP pathway may help us to prevent BE and subsequently the highly malignant BE associated esophageal adenocarcinoma.

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Chapter 7

Summary, General discussion
and future perspective
Summary

Esophageal Adenocarcinoma (EA) is an extremely aggressive cancer, with the most rapidly rising incidence in the Western World. At presentation approximately half of the patients with EA have aggressive and incurable disease, and the other half will undergo surgery. Despite surgical with or without (neo) adjuvant treatment this patient group has a poor prognosis with a five years survival rate of less than 20%. For this reason, alternative and more efficient treatment strategies are urgently requested. Immunotherapy using Dendritic Cells (DC) has been used for treating several aggressive cancers, such as melanoma and prostate cancer. The principle of the therapy is similar to that of conventional antiviral vaccines, using DCs as the vehicles. These are the most potent antigen presenting cells (APC) of the immune system, with the unique ability to induce a primary immune response, thus permitting establishment of immunological memory. DCs are highly capable of inducing T-cell responses, and the interaction of these two cell types, is determinant for the type of immune response that will be raised. The capacity of the DCs to stimulate T-cells depends on many factors, such as surface expression of co-stimulatory molecules, and the cytokine microenvironment. Functional DCs can be generated from human peripheral blood monocytes and be further matured into DCs that in turn can be used as vaccines for treating malignancies. To generate a cytotoxic T-cell (CTL) response against tumor cells, specific tumor antigens have to be presented to T-lymphocytes by immuno-activatory DCs. Different antigens, such as synthetic peptides, tumor lysates, and cDNA or RNA encoding for specific tumor-associated antigens as well as total tumor mRNA, have been used and tested for their immunopotency. DC immunotherapy has been used in many clinical trials for treating cancer patients, particularly for melanoma and prostate cancer. Although DC therapy holds a promise for the future as a potent patient tailored approach for curing cancer, there are several caveats in this field. To further optimize DC therapy for treating EA patients, this thesis addressed several of these difficulties. Moreover, two important novel protocols aiming on more potent DCs and for developing an improved ex-vivo read out system have been established. In addition, more insight has been gained in the immunogeneic tumor environment, and finally an important combinatorial treatment regiment has been explored.

The first part of the thesis describes a novel method that was used to obtain mature antigen presenting immuno-stimulatory DCs (chapter 2). Here the novelty is the use of monocytes, freshly isolated from peripheral blood, as compared to immature DCs, classically used for the loading of antigens, for the preparation of the vaccines. Of interest is that the yield of mature DCs finally presenting antigens, still after 8 days by using monocytes, is significantly higher as compared to the use of immature DCs, which are as well less potent, as shown
in the paper. The electroporated monocytes that matured into DCs, were tested for their immuno-phenotype, antigen presentation capacity, migration properties, and T-cell stimulation ability, and resulted to be of higher quality as compared to immature DCs used for loading of antigens. This method that resulted to be valid to produce mature immuno-potent DCs, was applied to patient material in chapter 3. In this chapter, the most important novelty is the establishment of a novel read out system for testing immune responses (T cell response). The model consists of primary cultures established from fresh biopsies isolated from EAC patients. Here, monocytes freshly isolated from EAC patients, were loaded with total tumor RNA isolated from biopsies, as well as normal RNA (for control purposes), which were as well used for obtaining the primary cultures. These monocytes were stimulated by different cytokines to be matured into DCs, and subsequently co-cultured with autologous lymphocytes for obtaining a population of lymphocytes containing several CTL populations against different tumor antigens. In the mean while the primary cultures were grown, and then co-cultured with the lymphocytes obtained in the previous phase. The response of the lymphocytes stimulated by either normal or tumor RNA loaded DCs, was observed particularly against autologous tumor cells, but not against normal cells, demonstrating that, first, this strategy is valid to monitor CTL responses against tumor cells, that can be tested ex-vivo in primary cell cultures. Furthermore, this approach does not induce adverse undesirable effects. This system can be used as a valid read out for preclinical evaluation of tumor cells response and could be used to predict efficacy of the DC vaccines. In the next part of the thesis, chapter 4, another important issue has been remarked: so far, the outcomes of patient treated by DC immunotherapy are extremely disappointing and below expectations. One of the reasons, which has been addressed for this phenomenon, is the unfavorable tumor microenvironment, which is often characterized by the presence of molecules, such as tumor growth factors and immunosuppressive cytokines, which inhibits the immune-response raised against cancer. This concept prompted us to gain insight in the EAC microenvironment, to be able to find out whether in this cancer, as in many others, the immune-response can be hindered by the presence of factors, which are immuno-suppressive. In this study, it was demonstrated that EAC, as many other cancer, is characterize by the expression of several molecules, such as COX-2, VEGF, TGF-β, IL-8, IL-1β, and many others, which exert either immunosuppressive function or tumor growth functions. These findings are of interest to establish, for instance, combinatorial treatments regiments using DCs immunotherapy, and targeting one or more of these molecules, in order to enhance the efficacy of the DC approach, and to increase patient outcomes. One other approach which is interesting for enhancing the efficacy of DCs immunotherapy is the use of this approach in combination with drugs, such as
monoclonal antibodies, directed against tumor antigens. One very interesting example is the monoclonal antibody directed against the tumor antigen HER-2, which is highly overexpressed, mainly via gene amplification, in several aggressive cancers. This antibody has already been used as treatment for breast cancer patients, and it has shown to have tumor growth inhibitory effects both in vitro and in vivo. EAC patients present HER-2 overexpression in 30 to 80% of the cases. For this reason, the approach of combining DC immunotherapy and the use of Trastuzumab was used and this is described in chapter 5. In this chapter, three different EAC cell lines, OE33, OE19 and Bic-1, were first characterized for the HER-2 status by FISH and ICC, and used to study the biological effect of Trastuzumab by performing an MTT assay. Once established that OE33 and OE19 were HER-2 positive, and Bic-1 HER-2 negative, and that Trastuzumab exerted highly inhibiting effects on OE33 and OE19 (HER-2 positive), but not on Bic-1 (HER-2 negative), a cytotoxicity assay was performed to assess whether the use of Trastuzumab could enhance the cytotoxic response induced by HER-2 specific CTLs against the two HER-2 overexpressing EAC cell lines. It is known indeed that Trastuzumab exerts its function through different mechanisms, which are basically induction of cell growth, activation of pathway which are controlling cell apoptosis, but also mechanism which are enhancing the immune-system, such as Antibody Depend Cell-mediated Cytotoxicity. It is hypothesized that Trastuzumab induces receptor internalization and degradation, therefore, increasing the volume of antigens loaded onto the MHC class I complex. This would induce an enhancement of the epitopes presented to the CTLs. EAC cell lines were treated with Trastuzumab. At the same time, HLA-A2 matched donors were used to isolate monocytes to load them with HER-2 RNA, mature these into potent APCs to stimulate the autologous lymphocytes and obtain HER-2 specific CTLs. These were subsequently co-incubated with the three cell lines either pretreated or not treated with Trastuzumab. In addition, PBMCs from EAC patients with HER-2 amplification were tested to investigate whether these patients could present self circulating HER-2 specific CTLs able to respond to the Trastuzumab pretreated cancer cell lines. It was remarkable to note that the percentage of cytotoxicity mediated by the HER-2 specific CTLs was significantly higher in the HER-2 positive cell line OE33 as compared to the HER-2 negative Bic-1, and this effect was even more significantly different when cells were pretreated with Trastuzumab. In addition, PBMCs isolated form EAC patient, which present HER-2 amplification in their cancer induced a significantly higher cytotoxicity effect on the cell line OE33 as compared to PBMCs isolated by EAC patients without HER-2 amplification. In the last part of the thesis there is more focus on the genesis of EA. In chapter 6 the role of a factor, the Bone Morphogenetic Protein-4 (BMP-4) is described. This gene was previously found to be significantly higher overexpressed in BE as compared to normal tissues by
Summary, general discussion and future perspective

Serial Analysis of Gene Expression. BE is a premalignant condition of the distal esophagus and consist in a metaplastic change of the normal lined squamous epithelium into a columnar type of epithelium, as a result of chronic longstanding gastro-esophageal reflux disease (GERD). Patients with BE have a significantly increased risk to develop EAC. In the last 3 decades, the incidence of BE and its associated EAC has increased and exceeds that of other malignancies. Therefore, when studying EAC it is as well of interest to gain further insight in the mechanism of development of BE which is the cause of EAC. In this chapter, we investigated whether BMP-4 and BMP pathway plays a role in the metaplastic transformation of normal squamous epithelium towards a columnar type of mucosa. To this aim, tissues from patients and an esophagitis-BE rat model were employed and investigated. Results showed that the BMP pathway is activated in esophagitis and BE. In addition, primary cell cultures of normal keratinocytes, obtained from biopsies, treated with BMP-4 showed activation of the BMP pathway by up-regulation of PhosphoSmad 1,5,8 and ID2 (BMP downstream targets). This effect could be blocked by pre-treatment with Noggin, an antagonist of BMP. In addition, the cytokeratin expression patterns and gene expression profiles were examined. BMP-4 treatment of keratinocytes resulted in shift of the cytokeratin expression pattern towards that of BE. In terms of gene expression, BMP-4 treatment resulted in a shift in the gene expression pattern of the normal keratinocytes of 26% towards that of BE. These results suggested that BMP-4 is involved in the process of transformation of the normal squamous epithelium into a columnar type of epithelium.

General discussion and future perspectives

The work described in this thesis focuses particularly on finding alternative and novel stratagems for treating esophageal adenocarcinoma patients and to improve the survival of this group of patients, which is considerably short and modest. Several approaches have been investigated in the past, including adjuvant and neo-adjuvant therapies, such as chemotherapy and radiotherapy, and the combination of these with surgery. The major problem for these patients is that at presentation they often carry an already advanced disease, therefore leaving extremely low prospective of life. This is the reason why the achievement of this thesis was to explore innovative approaches to create different more effective strategy and improve the life expectancy for this patient population. One tremendously appealing strategy that has been applied as an attempt to cure several aggressive cancer is DC immunotherapy. Therefore this was studied in this thesis, and several findings have been accomplished. Important findings are reported about the establishment of an efficient method to obtain sufficient
amount of antigen presenting DC using total RNA as loading strategy. This method would overcome the enormous amount of cell lost which is determined by the laborious protocol that is currently used to prepare mature antigen presenting DCs e-vivo. Using our approach, in which monocyte are first electroporated and then further matured towards DCs, we were able to obtain higher yields immune-stimulatory DCs as compared to the most used approach of loading immature DC. This is of importance since one of the difficulties correlated to this therapy is the possibility to obtain sufficient amount of DCs to be given to patients. This approach is effective as well when using esophageal cancer patients monocytes, which result in satisfactory amounts of immuno-potent DC. The effectiveness of these cells to induce an immune response was tested ex-vivo using autologous primary cultures. This approach gives the remarkable possibility of testing the potential patient response in vivo, by using a novel autologous ex-vivo system. The feasibility of this autologous read out system to evaluate cytotoxicity responses against esophageal cancer cells is reported, next to the necessity to evaluate the concern of breaking tolerance to self-antigens, when immunizing with total tumor RNA. The use of non autologous systems can indeed induce to over evaluation of the possible results when looking at immune responses, therefore, an autologous read out might be of use, especially as preclinical tool to predict potential immune-response, or adverse effect, in a patient tailored approach. Numerous studies attempted at the development of very effective and safe DC vaccines, and this was partly successfully reported by several groups. However, patient who underwent this treatment did not show remarkable outcomes up to now, inducing the disappointment of the researcher and clinicians. The expectancy in terms of anti-cancer immune responses was significantly higher, considering the abundant highly promising results obtained in vitro, in terms of immune-response. Therefore it was required to address an explanation for this phenomenon. Finally few research groups emerged with a very essential issue: the tumor microenvironment, constituted by a complex network of cytokines, growth factors, and different cell types, is determinant for an appropriate immune-response in an immuno-competent host. These reports prompted to novel studies, based on a deeper and more profound understanding of the interactions, and the mutual influences, of cells, cytokines, and growth factors. In this thesis the answer to this issues, regarding particularly the esophageal adenocarcinoma, is given by the description of expression of several cytokines, growth factors, angiogenic factors, and immuno-suppressive factors which characterize the tumor surrounding in esophageal cancer patients. This part of the study provides an overview of the most important cytokines and tumor growth factors pattern of expression in esophageal cancer, and therefore can be of use for introducing novel approach based on targeting these molecules in order to overcome the immuno-suppressive effect that they mediate. Despite
many efforts and several important achievements, the field of immunotherapy needs to be further explored. In particular, several issues has to be taken into account, like, for instance, the discrepancy between the in vitro data, highly promising, and the clinical outcomes, still below expectations, and limited to a small amount of patients. Further studies will have to be performed to establish to which extent the tumor microenvironment interferes with the functions of the immune system, permitting tumor escape from immunity. Therefore, more evaluation will be carried out. In future approaches, it is proposed to focus the attention not only towards one single strategy, but the combination of different stratagems, which could lead to more satisfactory results. Nowadays several approaches have been combined, like, for instance, surgery with chemo, and/or radiotherapy, and surgery with drugs, such as antibody and small molecules. To this point, we conducted a study in order to establish if such a strategy could be of interest, and, above all, applicable, to esophageal adenocarcinoma, therefore providing another alternative possibility of cure for this cancer. The use of antibodies in clinics is currently a common approach, based on targeting particular molecules which characterizes certain type of cancers. The HER-2 gene is a very well know oncogene, which is already used as target for the therapy of HER-2 overexpressing cancer, such as breast cancer. Esophageal cancer part of this group, with 50% of the cases bearing HER-2 gene amplification, would result as a suitable candidate for such a strategy. Targeting HER-2 with the use of a specific antibody of HER-2, Trastuzumab, which represent a therapy for HER-2 overexpressing breast cancers, was tested in vitro in esophageal cancer cells, used in combination with DC immunotherapy. Encouraging results elucidated the potential role of the antibody, in determining an improvement of T-cell responses against cancer cells mediating an Antibody Dependent Cellular Cytotoxicity mechanism. The mechanism of action of this particular antibody is not yet well understood; therefore further research is needed in order to achieve a more profound comprehension of this type of therapy, in terms of efficacy, but as well safety, for patients. Other similar approaches have been investigated, and these are the use of small molecules, such as tyrosine kinase inhibitors. Of interest is the fact that those have been already tested, with perhaps hopeful results, although one common features of this type of approach, is the heterogeneity of the patients response. Based on these observations, it is anticipated that in the future, curative treatment for cancer will become patient tailored, to ensure a more successful result. This concept is of high interest, because is taking into account the extreme diversity of patient even though belonging to the same group of disease.

Of outmost importance is certainly the exploration of earlier phases of this type of cancer development, which lead to the final aggressive adenocarcinoma, and this is the awareness of the Barrett’s metaplasia. It is still not know what the exact molecular mechanism behind the development of the Barrett’s metaplasia
is and the consequent progression to a malignant disease. The last part of this thesis is a “back to the origin” investigation, which focused on the role of Bone Morphogenetic Protein 4, a morphogen found to be overexpressed in Barrett’s esophagus as compared to normal controls tissues, in the transformation from the normal squamous to a columnar type of epithelium characteristic of Barrett’s esophagus. It was ruled out that this molecule is certainly involved in this process of transformation towards a un-specified columnar type of epithelium, certainly in the primary stages, but still a brick is missing in the wall: what determines the final transformation to a specialized columnar type of epithelium, such as the one characteristic of Barrett’s esophagus? This point needs to be clarified by performing additional studies in order to further delineate the molecular mechanism lying behind the development of Barrett’s esophagus.

This work provides a number of answers, and offer some advances, but consequently enlightens new questions, contributing to the intriguing never-ending search of answers and progresses and prompting further investigations to improve patients outcomes when attempting to combat cancer diseases.
Appendices
Samenvatting

Adenocarcinoma van de slokdarm is een extreem agressieve kanker en kent, in de Westerse wereld althans, de snelst stijgende incidentie van alle kankers. Op dit moment blijft in ongeveer de helft van alle patiënten met adenocarcinoom van de slokdarm de ziekte onbehandelbaar terwijl de andere helft operatief wordt behandeld. Desalniettemin kent ook dit laatste cohort een slechte prognose, met een vijfjaars overleving na diagnose van minder dan 20% in deze patiëntengroep. Het moge duidelijk zijn dat we dringend behoefte hebben aan nieuwe meer efficiënte behandelwijzen.


Het maken van dergelijke vaccins is echter niet zo simpel. Adenocarcinoma van de slokdarm verschilt van samenstelling van patiënt tot patiënt, wat het ontwikkelen van dergelijke vaccins niet helpt. Bovendien is nog slecht uitgezet hoe men het effectiefst dendritische cellen aanzet om tumor materiaal aan het immuun systeem te presenteren. Ook is de lokale immunologische omgeving van adenocarcinoma in de slokdarm nog maar slecht in kaart gebracht, terwijl kennis hiervan noodzakelijk is om effectieve anti-kanker vaccins te ontwikkelen. Tenslotte weten we nog maar weinig hoe het combineren van verschillende behandelingen ons kan helpen bij het bestrijden van slokdarmkanker. Het onderzoek beschreven in dit proefschrift was opgezet deze barrières tegen het ontwikkelen van effectieve anti-kanker vaccinatie uit de weg te ruimen en heeft, nu aan het eind van de rit, er toe geleid dat de toekomst van een dergelijke strategie er rooskleurig uitziet.

Effectieve productie van verbeterde dendritische cellen

Een eerste hindernis op weg naar effectieve vaccinatie tegen slokdarmkanker vormt het verkrijgen van effectieve dendritische cellen. In hoofdstuk 2 wordt deze barrière genomen. Dendritische cellen ontstaan uit de opeenvolgende ontwikkeling van monocyten (een celtype dat in grote aantallen in ons bloed aanwezig is) naar onvolgroeide dendritische cellen om uiteindelijk de volwassen dendritische immuun-stimulatoire effector cellen te vormen. Klassiek werden
Samenvatting

voor het maken van kanker vaccins altijd onvolgroeide dendritische cellen opgeladen met tumorcomponenten, na doorgroei tot volwassen dendritische cellen zouden deze componenten dan aan het immuunsysteem gepresenteerd worden. In dit hoofdstuk laat ik zien dat het opladen van monocyten een veel effectievere strategie is, wat de basis vormt voor het verdere werk beschreven in dit proefschrift.

**Dendritische cellen zijn in staat om in het immuunsysteem een anti-slok darmkanker antwoord op te wekken**

In *hoofdstuk 3* worden dendritische cellen gemaakt uit het bloed van slokdarmkanker patiënten en worden deze cellen opgeladen met tumor materiaal uit dezelfde patiënt, waarna buiten het lichaam het vermogen van deze cellen om een anti-kanker antwoord van het immuunsysteem op te wekken wordt getest (met behulp van immuun cellen en cultures van het adenocarcinoma van de patiënt). Het blijkt dat de dendritische cellen krachtige anti-tumor activiteit opwekken, de slokdarmkanker kweken worden efficiënt gedood door de met dendritische cellen gestimuleerde immuuncellen. Kweken van normale slokdarm van deze patiënten worden echter met rust gelaten. Deze proeven vormen een bewijs dat anti-tumor vaccins in ieder geval onder laboratorium omstandigheden werken.

**Hoe zit het met de tumor omgeving?**

Eerdere experimenten met anti-kanker vaccins waren teleurstellend. Vaak wordt gesuggereerd dat de tumor een omgeving voor zich zelf creëert die ongunstig zou zijn voor het immuunsysteem. Deze kwestie wordt onderzocht in *hoofdstuk 4*. Hiertoe werd de omgeving van het adenocarcinoma van de slokdarm nauwkeurig in kaart gebracht. Het bleek inderdaad dat verschillende immuunsysteem onderdrukkende factoren aanwezig waren, samen ook met groeifactoren voor deze kankers. Kennis van deze factoren stelt ons in staat om tumor vaccins te combineren met andere interventies speciaal gericht om de voor de slokdarmkanker zo gunstige omgeving minder aangenaam te maken.

**Combinatietherapie lijkt meer effectief**

De bovengeschetste mogelijkheid wordt direct getest in *hoofdstuk 5*, waar het vermogen van de HER-2 antagonist Trastuzumab werd getest. Inderdaad bleek dat in HER2-positieve kankercellen Trastuzumab de effectiviteit van het immuunantwoord kan versterken. Ofschoon deze experimenten onder tamelijk kunstmatige omstandigheden werden uitgevoerd, lijken zij wel aan te tonen dat combinatietherapie de aangewezen weg is.
Samenvatting

**Beter inzicht in het ontstaan van adenocarcinoma van de slokdarm**
Het ontwikkelen van nieuwe therapie is uiteraard ook gebaat bij beter inzicht van hoe deze gevaarlijke ziekte tot stand komt. Het is bekend dat de ziekte ontstaat uit zogenaamd metaplastisch epitheel: normaal is het slokdarmepitheel (de eerste barrière tussen darminhoud en het lichaam) een meerlagig gebeuren, waarin langgerekte cellen liggen. Gedurende het voorstadium van adenocarcinoma verandert deze laag in een enkelvoudig rechthoekig epitheel, iets wat we Barrett’s oesofagus noemen. In **hoofdstuk 6** identificeren we het hormoon dat deze overgang bewerkstelligt. Uiteraard biedt dit ons nog aangrijpingspunt voor betere preventie en behandeling van het adenocarcinoma van de slokdarm.

**Samenvattend…**
Dit proefschrift geeft nieuwe antwoorden op een aantal vragen waarmee reeds lang geworsteld werd m.b.t. het ontwerpen van nieuwe therapie voor het adenocarcinoma van de slokdarm. De volgende stap zal nu een fase 1 studie waarin onze methodologie direct op de patiënt getest zal worden.
Dankwoorden

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Dankwoord

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Francesca
Dankwoord
List of Publications

An improved Protocol for generation of immuno-potent DC through direct electroporation Of CD14+ monocytes.

An ex-vivo read out for evaluation of Dendritic cell induced autologous CTL responses against esophageal cancer.


A comparative analysis by SAGE of gene expression profiles of Barrett’s esophagus, normals quamous esophagus, and gastric cardia.


Efficient automated assessment of genetic abnormalities detected by Fluorescent In Situ Hybridization on brush cytology of a Barrett’s esophagus surveillance population.

Comparison of kinome profiles of Barrett’s esophagus with normal squamous esophagus and normal gastric cardia.
Curriculum Vitae

Francesca Milano was born on April 20, 1976 in Perugia, Italy. She graduated from highschool in 1995 at the Liceo Classico Mariotti in Perugia. In 1995 she started biology school at the University of Perugia. In 2002 Francesca graduated in medical biology in Perugia, and, in the same year, she was granted with a research scholarship at the Netherland Cancer Institute, in Amsterdam. Therefore she moved to Holland and, under the supervision of DR. D. Weinkove, she studied the role of Ppk-2 and Daf-16 in the oxidative stress in Caenorabditis Elegans. In 2003 Francesca started her PhD project at the Laboratory of Experimental Internal Medicine and Experimental Gastroenterology in the Academic Medical Center, in Amsterdam. Under the supervision of Dr. K.K. Krishnadath and Prof. M.P. Peppelenbosch, she conducted her research directed to find novel and more effective strategy to treat Esophageal Adenocarcinoma patients, particularly focusing on Dendritic Cell immunotherapy, and to study the molecular mechanism underlying the cause which is at the origin of this type of cancer, which is Barrett’s Esophagus. The results obtained so far are reported in this thesis. Currently she is continuing her studies on how to use Dendritic Cells immunotherapy in combination with other approaches, such as the use of small molecules inhibitors and blocking antibodies, in order to improve patient’s outcomes. Furthermore, she is further characterizing the dynamics of the development of Barrett’s esophagus.