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Combination of immunotherapy with anaerobic bacteria for immunogene therapy of solid tumours

Research Article

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Summary

Solid tumours possess unique microenvironment characterised by defective vessels, heterogeneous tumour cell, hypoxic regions, and anaerobic metabolisms. These often become intrinsic and acquired barriers to current therapeutical approaches, but they also create an ideal condition for the growth of anaerobic bacteria, which have shown specificity in their germination and multiplication. Spores from the strictly anaerobic clostridial had demonstrated ability in tumour specific colonisation and induction of tumour lysis following intravenous delivery. Clostridial strains genetically modified to act as "Trojan horse" gene therapy vectors have been developed. Similarly, recent development in immunotherapy strategies for cancer also utilizes gene transfer to facilitate a dormant host immune response directed against the tumour. Combination of anaerobic bacteria for cancer gene therapies with immunotherapy will probably be the most promising approach that can potentially generate a prolonged anti-tumour effect beyond the immediate treatment period of gene therapy, allowing for treatment of advanced primary tumours and disseminated disease. In this review, we introduce the recent understanding of tumour microenvironment and detail the advances in the use of anaerobic bacteria for cancer gene therapies and recent studies in immuno therapy for cancers. We believe that the use of combined treatment modalities of such will provide a rational paradigm to improve upon the clinical efficacy of cancer therapy.

I. Introduction

Cancer is one of the major health problems of mankind, accounting for 7.6 million of death world wide. Cancer mortality is expected to increase further, with an estimated 9 million people dying from cancer in 2015. This figure will rise to 11.4 million in 2030 (WHO 2006) (Cho, 2007).

Of all cancer diagnosed, 90% of these are solid tumours. As they do not have particular noticeable symptom or signs for early detection, a significant percentage of the patients with newly diagnosed disease have regional or advanced, inoperable disease, especially in developing countries where diagnostic facilities are suboptimal. Conventional therapies include surgical operation, radiation and chemotherapy. Single or a combination of methods may be used, depending on various factors such as the type and location of the cancer. Unfortunately, current cancer treatments are limited to effect. Furthermore they also cause severe side effects. The search for new cancer therapies is one of the most pressing tasks of medical science.

Cancer development results from constant battle between tumour cells and host defence system. Once it establish by itself. Its microenvironments are hostile to therapeutic including immunotherapy as well as gene

therapy. In this paper, we review current understanding of tumour microenvironments and recent advances in therapy of solid tumour and explore potential combinations of immunization and anaerobic bacteria for cancer management.

II. The unique microenvironment of solid tumours

A. Overview

All solid tumours, when they grow more than 2 mm diameter in size, undergo angiogenesis that results in biological changes and adaptive metabolisms, i.e.: formation of defective vessels, appearance of hypoxic areas, and emergence of heterogeneous tumour cell population. Thus, solid tumours are organ-like structures that are heterogeneous and structurally complex, consisting cancer cells and stromal cells (i.e., fibroblasts and inflammatory cells) that are embedded in an extracellular matrix and nourished by a vascular network; each of these components may vary from one location to another in the same tumour. Compared with normal tissues, the tumour stroma is associated with an altered extracellular matrix and an increased number of stromal that synthesize growth factors, chemokines, and adhesion molecules (Aznavoorian et al, 1990). The extracellular matrix can vary greatly among tumours, both in amount and in composition (Ohtani, 1998). Also the tumour stroma can influence malignant transformation (Tlsty 2001) plays an important role in the ability of tumours to invade and metastasize and affects the sensitivity of tumour cells to drug treatment. The amount composition and structure of stromal components in tumours also contribute to an increase in interstitial fluid pressure, which hinders the penetration of macromolecules through tissue (Croker, 2008). Also, the three-dimensional structure of tissue itself can influence the sensitivity of constituent cells to both radiation and chemotherapy (Shicang 2007).

B. Tumour vasculature and blood flow

Solid tumours at advanced stages have abnormal vasculature, which influences the sensitivity of the tumour to therapies. Anticancer drugs gain access to tumours via the blood and limited supply of nutrients in tumours leads to metabolic changes (including hypoxia) and gradients of cell proliferation that influence drug sensitivity (Tatum et al, 2006). Also, blood vessels in tumours are often dilated and convoluted. Compared with normal tissues, tumour blood vessels have branching patterns that feature excessive loops and arteriolar–venous shunts, in some tumours they are not organized into arterioles, capillaries, and venules but instead share features of all of these structures. The walls of tumour vessels may have fenestrations, discontinuous or absent basement membranes that may lack perivascular smooth muscle (Hallmann et al, 2005) and fewer pericytes than walls of normal vessels. In addition, cancer cells may be integrated into the vessel wall. These abnormalities tend to make tumour vessels leaky, although their permeability varies both within and among tumours.

C. Tumour hypoxia and acidity

Most solid tumours contain regions of hypoxia (Wu et al, 2006). The limited vasculature of tumours results in insufficient blood supply and chronic or diffusion-limited hypoxia. Tumour cells in hypoxic regions may be viable, but they are often adjacent to regions of necrosis. Tumour cells in regions proximal to blood vessels can migrate into hypoxic areas and become necrotic, presumably because of nutrient deprivation. If cells close to blood vessels are killed by treatment, the nutrient supply to previously hypoxic cells may improve, allowing those cells to survive and regenerate the tumour (Trédan et al, 2007). Transient hypoxia is also common in tumours and results from the temporary shutdown of blood vessels. Hypoxic regions of tumours are likely to have a decreased supply of nutrients such as glucose and essential amino acids (Pouysségur et al, 2006). The presence of hypoxia in tumours is known to lead to the activation of genes associated with angiogenesis and cell survival that is mediated by the transcription factor hypoxia-inducible factor 1(Bos R et al, 2004). Expression of these genes may result in the expansion of populations of cells with altered biochemical pathways that may have a drug-resistant phenotype. Transient hypoxia has been reported to cause amplification and increased expression of the genes encoding P-glycoprotein and dihydrofolate reductase, which induce drug resistance to substrates of P-glycoprotein and to folate antagonists, respectively. Transient hypoxia that is associated with glucose deprivation can also disrupt protein folding in the endoplasmic reticulum; this effect may confer resistance to topoisomerase II–targeted drugs and enhance P-glycoprotein expression and multidrug resistance (Chen et al, 2003).

The pH in the tumour microenvironment can influence the cytotoxicity of anticancer drugs (Philip et al, 2005). Molecules diffuse passively across the cell membrane most efficiently in the uncharged form. The extracellular pH in tumours is low and the intracellular pH of tumour cells is neutral to alkaline, weakly basic drugs that have an acid dissociation constant of 7.5–9.5 are protonated and display decreased cellular uptake. Alkalinization of the extracellular environment enhances the uptake and cytotoxicity of some of these drugs (Trédan et al, 2007). By contrast, weakly acidic drugs concentrate some in the relatively neutral intracellular space. The acidic microenvironment may also inhibit active transport of some drugs (Mahoney et al, 2003).

D. Tumour immunosuppression

During the constant battle between tumour and immune system, tumour cells developed multiple ways to fight back the immune system.

1. Avoidance of effectors T cell killing

One of well established strategies is down regulation of antigen presentation by tumour cells, especially through MHC class I restricted antigen presentation pathway. Tumour cells can down regulation, even loss of MHC class I molecules on their cell surface (Frey, 2006), mutation of proteins associated with this pathway, such as TAP and LMP2 and LMP7.

Tumour or stromal cells also secrete factors that damp immune responses. TGF (tumour growth factor), IL-10 are two cytokines with immune suppressive functions usually found with high levels within tumour. TGF levels are associated with poor prognoses of cancers including prostate, gastric and bladder carcinoma (Biswas et al, 2007). TGF inhibits T cell activation and differention of cytotoxic T cells and promotes NKT cells mediated inhibition of CTL responses together with IL-13 (Biswas et al, 2007). IL-10 down regulate antigen presentation by dendritic cells and promote the generation of Tr1 regulatory T cell generation (Suciu-Foca et al, 2003) and Inhibit CTL response in antigen experienced host (Tamada et al, 2002). High levels of prostaglandin E2 (PGE2) have been shown in colorectal, lung and bladder cancer (Akasaki et al, 2006). It has been demonstrated that PGE2 promotes the generation of IL-10 secreting CD4 T cells through the induction of IL-10 secreting dendritic cells (Cools et al, 2007).

Different tumour types have also been expressed PD-L1, an immune suppressive molecule. Tissue histology study showed that freshly isolated carcinomas of human lung, ovarian, colon, melanoma, head and neck cancers, and breast cancers can express PD-L123. PD-L1 a suppressive molecule, engagement of PD-L1 with PD-1 of effector T cells causes T cell apoptosis (Yang et al, 2008). B7-H1 positive melanoma cells were also more resistant to specific CTL, while nearly all B7-H1 negative tumour cells were eliminated in the cultures (Dong and Chen, 2003), these results suggest that expression of suppressive molecule is another strategy used by tumour cells to avoid from killing by effector cells.

2. Regulation of immunoresponses by regulatory T cells

Regulatory T cells are groups of T cells that regulatory immune response, different compartments of T regulatory cells including CD4+, CD8+ and NKT cells have been identified. CD4+CD25+ Foxp3+ thymus derived T regulatory cells and antigen induced IL-10 secreting CD4 T cells are the 2 main types identified. NKT cells have also been shown to have regulatory function during tumour development (Berzofsky et al, 2008). However, the number of T regulatory cells with human ovary cancer is related to poor prognosis of cancer (Koido et al, 2005). Also, it has been shown that myeloma cells promote the generation of IL-10 secreting Tr1 T cells (Battaglia et al, 2006). Tr1 cells can be isolated from tumour infiltrating lymphocytes in B16 tumour model (Seo et al, 2001). Human bladder cancer tissues contain high number of Foxp3+ cells and mRNA level of IL-10 (Petrulio et al, 2006). It is not clear whether the T regulatory cells were boosted from existing T regulatory cells or vaccine induced.

However, immunotherapy has shown to amplify tumour specific T regulatory cells, thus impede effective immunotherapy in a mouse tumour model (Reilly et al, 2000); moreover, similar results were also observed clinically. Patients with resected HPV16-positive cervical cancer were vaccinated with an overlapping set of long peptides comprising the sequences of the HPV16 E6 and E7 oncoproteins emulsified in Montanide ISA-51. The vaccine-induced responses were dominated by effector type CD4(+)CD25(+)Foxp3(-) type 1 cytokine IFN gamma-producing T cells but also included the expansion of T cells with a CD4(+)CD25(+)Foxp3(+) phenotype (Welters et al, 2008).

3. Abnormal antigen presentation cells

Antigen presentation cells include dendritic cells (DC), macropaghes and B cells. Matured DCs play key roles for the priming of naive T cells, including CD8+ T cells, which is critical for the killing of tumour cells. Tumour microenvironments usually have less functional competent matured but more immature DCs, which can not effectively activate T cells. Furthermore, it has been reported that in tumour tissues, there are subset of DCs that suppress T cell function. This T cell suppression has been shown in cancer patients as well as animal tumour models.

Immune cells in the tumour microenvironment are dysfunctional, generally fail to control tumour growth and may even promote its progression. Molecular mechanisms responsible for tumour-induced local and systemic immune suppression are currently under intense discussed. It appears that tumours can deregulate recruitment, effector functions and survival of immune cells, interfering with all stages of antitumour response. Suppressive mechanisms targeting key signalling pathways in immune cells have been identified. Strategies for reversal of tumour-mediated immunsuppression are being developed. Confirmation of multiple and varied mechanisms used by tumours to escape immune surveillance is crucial for the future design in antitumour therapies.

III. Current cancer gene therapy and immunotherapy approaches

A. Current development in gene therapy of solid tumour

Cancer is, at present, the disease most frequently targeted by gene therapy because its promise of potential for selective potency. To achieve this aim, cancer gene therapy strategies attempt to exploit the biological uniqueness of each particular tumour. Cancer gene therapy may be defined as the transfer of recombinant DNA into human cells to achieve an anti-tumour effect. Gene therapy will have a major impact on the healthcare of our population only when vectors are developed that can

safely and efficiently be injected directly into patients as drugs. One of the most strategies of vector development is that of non-viral vectors, which consist of liposomes, molecular conjugates, and naked DNA delivered by mechanical methods. The modifying viral vectors should be focused to reduce toxicity and immunogenic, increasing the transduction efficiency of non-viral vectors, enhancing vector targeting and specificity, regulating gene expression, and identifying synergies between gene-based agents and other cancer therapeutics. A universal gene delivery system has yet to be identified, but the further optimization of each of these vectors should result in each having a unique application.

1. Pro-Drug activation vectors

Several experimental models relying on pro-drug activation vectors (Kanai et al, 2008). One such a model involves local injection of gene therapy vectors into tumour sites. This model may benefit from the so-called "bystander effect," a reflection of the biological observation that pro-drug activation to 5-fluorocysteine (5-FU) releases this chemotherapeutic not just in the tumour cells, but in the surrounding cell environment as well. In fact, using in vitro systems, it has been found that only 5% of tumour cells need to be infected by the delivery vector for anti-tumour effect to be seen throughout the whole tumour cell population. An adenovirus vector expressing the cytosine deaminase enzyme will be injected into the prostate bed using similar techniques as those now used for radiation implants. These patients will then be given the pro-drug, which in principle will be activated to 5-FU in the prostate gland. This should allow localized cytotoxic therapy to the prostate and possible synergistic benefit between 5-FU and the concurrent radiation therapy.

The other model system which is used in clinical trials deals with autologous transplantation for metastatic breast cancer. In this system, harvested bone marrow is exposed to the viral vector, which infects the epithelial tumour cells efficiently, but normal marrow stem cells less efficiently. After intensive chemotherapy, patients are then given this modified marrow population. Once engrafted, patients are treated with the pro-drug 5-FC, which in principle should be toxic only to the infected tumour cells. This trial is open to women with known marrow involvement by tumour cells, and who are therefore not candidates for standard high-dose therapy.

2. Tumour-specific gene promoters

The L-plastin gene (Akbulut et al, 2003), as another means of conferring tumour-specific expression which encodes an actin-binding protein, show the new vector model with a tumour specific gene promoter. The estrogen-dependent tissues such as ovary and breast were selectively expressed in ovarian and breast cancer. The promoter for this gene is added to the adenoviral vector, and a reporter enzyme, such as beta-galactosidase, is linked to the promoter to allow for assessment of expression. In preliminary experiments, this vector was able to transfect ovarian cancer cells isolated from ascites fluid, and confer tumour-specific expression of beta-galactosidase. This method creates the possibility of targeting expression of certain genes in specific tissues

3. Herpes simplex virus thymidine kinase gene

To broaden the effect of gene therapy, vectors employing both the thymidine kinase gene and the genes for immunomodulatory cytokines such as IL-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF) have been developed (Iwadate et al, 1997). In mice, injection of these vectors into tumours and treatment with ganciclovir had both a direct anti-tumour effect in the liver, as well as a systemic effect in generating tumour-specific immune responses. As a result, these mice are resistant to subsequent tumour challenge. This system establishes the principle that localized gene therapy might ultimately have systemic protective or therapeutic effect by stimulating immune mechanisms which can act throughout the organism. A phase I trial for patients that would include treatment with a thymidine kinase and cytokine (IL-2) vector is being planned. The principle endpoint of the study will be the determination of an anti-tumour immune response.

4. Dendritic cells as targets for cancer gene therapy

DCs are the most potent APCs in the immune system and are central to the success of these genetically engineered tumour vaccine strategies. Activated DCs can present prostate tumour vaccine-associated antigens; they have processed to both CD4 (helper) and CD8 (cytolytic) T cells in the draining lymph node of the vaccination sites, activating a systemic tumouricidal immune response. The possibility of obtaining large numbers of DCs in vitro has boosted research on their ontogeny and functions. The unique ability of DCs to take up, process, and present antigens, and to activate naive $CD4^+$ and $CD8^+$ T cells, makes them appropriate candidates for the immunotherapeutic approach.

In a mouse model, DCs are harvested and then transfected with adenoviral vectors. These vectors expressed a foreign protein, beta-galactosidase. The dendritic cells were then injected into mice, and served to prime an immune response against that protein. This ex vivo gene therapy has many potential human applications. Three major myeloid DC populations have been identified in vivo: (1) epidermal Langerhans' cells (LC); (2) interstitial (or dermal) immature DC; and (3) mature interdigitating DC, found in secondary lymphoid organs. In the early stages of DC research, the limited accessibility of these cells in vivo as well as their difficult ex vivo culture hampered attempts to study this particular cell type in more detail. In the 1990s, this problem was solved by the efforts of various research teams which revealed the hematopoietic lineages through which DC differentiate,

and established in vitro expansion protocols to obtain sufficient quantities of DC for clinical use (Caux et al, 1992; Sallusto, 1994). The unique ability of DC to stimulate primary immune responses stems from several factors. The immature DC type uses elegant systems, including macropinocytosis, mannose receptor-mediated uptake, Fcg receptor III (FcgRIII)-mediated uptake and phagocytosis to efficiently take up exogenous antigens, either self or non-self, from the periphery (Steinman et al, 1999). After antigen capture, DC leaves the peripheral tissue and migrates via blood or lymphatic vessels to the draining lymph nodes where they activate T cells Given their central role in controlling immunity and their link with the innate immune system, DC are often called nature's adjuvant. Therefore, DC is logical targets for immunotherapy of cancer. The fact that tumours do not elicit a therapeutic T cell response may be due to the absence of competent DC at the tumour site.

B. Cancer gene therapy existing problems

Currently, there are many different approaches to fight cancer with gene therapy. Morgan et al report has revealed encouraging results for the use of gene therapy as a treatment for cancer (Morgan et al, 2006). However; two principal obstacles continue to limit further advances in gene therapy. The first is a technical problem, the development of an appropriate delivery system -- a reliable, safe, and effective means for introducing genetic material into the target cells or tissues. The second problem is a biological one -- developing an understanding of the molecular basis underlying cancer in order to determine where single alterations in genetic expression might allow effective anti-cancer therapy. In viral vector, the efficiency of transduction is not sufficient for therapeutic measures (Marina et al, 2003). One important parameter is whether the genetic alteration has to be lasting or temporary (stable or transient transfection). Of overall importance is the question of biological safety, which means that the vector itself does not create a novel threat to the patient's health. The key to a successful gene therapy is the vector system. Various vectors have been developed with unique features, including viral and non-viral based therapy systems (Wagner, 2007). However, due to the complex nature of cancers, these vectors suffer from several deficiencies: firstly the majority of vectors currently in use require intratumoural injection to elicit an effect, far from ideal as many tumours are inaccessible and spread to other areas of the body making them difficult to locate and treat. Second, most vectors do not have the capacity to efficiently enter and kill every tumour cell.

The emerging challenges of cancer gene therapy: i) which better route of administration is best for improving gene delivery; iii) optimizing new vector best suited to the target type of tissue and reducing toxicity, Although as with many gene-therapy approaches, considerable barriers will need to be overcome to make the technique more reliable and widely applicable - achieving long-term expression of therapeutic genes is a particular problem - these results are nevertheless a heartening 'proof-of-principle' demonstration of the potential power of gene therapy to combat cancers.

To establish efficient and safe gene delivery in vivo, a number of new techniques and concepts have been introduced with improvements in targeted or controlled delivery of genes. But we have come a long way in understanding the cellular barriers which prevent proper delivery of DNA or viral vectors. Cancer gene therapy has still a long way to go in the basic and clinical sciences.

C. Anaerobic bacteria for cancer treatment

Interest in microbe-based approaches to cancer therapy has recently re-emerged with the development of methods to genetically engineer bacteria, reducing their toxicity and arming them with genes encoding pro drug-metabolizing enzymes.

1. Anaerobic bacteria as tumour target vector

The unique solid tumour micro-milieu, though, provides a haven for anaerobic bacteria. Anaerobic and facultative anaerobes tested so far fell into three classes. (1) the lactic acid, Gram-positive anaerobic bacteria; (2) the intracellular, Gram-negative facultative anaerobes, and (3) the strictly anaerobic, Gram-positive saccharolytic/ proteolytic Clostridia. At the molecular level, bacterial infections like those of Clostridia novyi (C. novyi) are associated with the release of pathogen-associated molecular patterns (PAMPs) from bacteria and Hsp70 from necrotic cells (Gelman, 2003). Hsp70 induces maturation of DCs, professional antigen-presenting cells that are essential for the production of potent immune responses. PAMPs interact with Toll-like receptors, leading to up-regulation of costimulatory molecules such as CD40 and proinflammatory cytokines such as IL-12. These in turn induce the production of IFN-γ and initiate a Th1-dependent cell-mediated response, primarily affected by $CD8^+$ cytolytic T cells (Kay, 2001). The demonstration that $CD8^+$ T cells from C. novyi-NT-cured mice can confer adoptive immunity in a tumour-specific fashion is consistent.

Clostridium is strictly anaerobic, sporulating Gram-positive bacteria. This genus is one of the largest genera comprising of about 80 species. Up to 10 species of Clostridia have been studied and as strictly anaerobic bacteria they all require an anaerobic environment to grow but their oxygen tolerance and biochemical profile varies considerably among different species. Clostridial spores had been used to induce tumour lysis following intravenous delivery and shown a distinct advantage over Bifidobacterium and Salmonella in terms of easy production, hardy storage and impressive oncolytic effects. Both proteolytic and saccharolytic Clostridia have been tested for cancer therapy. When C. novyi-NT spores are injected intravenously into immunodeficient mice bearing human xenografts, the spores quickly germinate within necrotic regions of the tumours. Hypoxic and

necrotic regions are generally localized within the central parts of tumours, with well perfused tumour cells occupying the rim. Because of the exquisite sensitivity of C. novyi-NT to oxygen (Dang et al, 2001), bacterial germination and spread halt when the bacteria reach the well oxygenated rim. It was shown that conventional chemotherapy and radiation therapy could be used to destroy the well oxygenated cells in this rim, and that the combination of C. novyi-NT provided substantial antitumour activity in several xenograft models.

2. Anaerobic bacteria and immune response

C. novyi is well known for its capacity to induce massive leukocytosis and inflammation (Agrawal et al, 2004), whereas many other species of Clostridia do not induce this level of response. The inflammatory reaction is classic in many ways, including the observed increase in neutrophil-directed cytokines in serum and the cellular nature and time course of the infiltrate. The antitumour effects of inflammation are well documented. Systemically administered C. novyi-NT spores are distributed throughout the body, but due to their strict anaerobic growth requirements, germinate only within anoxic or markedly hypoxic regions of tumours. Once germinated, the bacteria destroy adjacent cancer cells through the secretion of lipases, proteases, and other degradative enzymes. At the same time, the host reacts to this localized infection, producing cytokines such as IL-6, MIP-2, G-CSF, TIMP-1, and KC that attract a massive influx of inflammatory cells, initiated largely by neutrophils and followed within a few days by monocyte and lymphocyte infiltration. The inflammatory reaction restrains the spread of the bacterial infection, providing a second layer of control in addition to that provided by the requisite anaerobic environment. The inflammation may also directly contribute to the destruction of tumour cells through the production of reactive oxygen species, proteases, and other degradative enzymes. Moreover, it stimulates a potent cellular immune response that can subsequently destroy residual tumour cells not lysed by the bacteria. The cure rate is determined by the balance between bacteriolysis, angiogenesis, regrowth of residual tumour cells, and the rate of development of the immune response.

During these years, bacteriological research on tumour associated anaerobic spore forming bacteria has accumulated a considerable amount of information and a variety of new concepts in experimental and clinical oncology (Agrawal et al, 2004). Of great importance was the systematic elucidation which convincingly demonstrated that the growth of anaerobes can be strictly interconnected with tumour growth. A whole series of experimental studies have been performed to elucidate the mechanisms which governed the selective, temporarily unrestricted clostridial growth and which formed the basis for the liquefaction of tumour tissue. Since tumour lysis with Clostridium oncolyticum spores is incomplete and, possibly, subject to non-specific systemic incompatibility [‘acute tumour lysis syndrome’]. Clostridia became significant in pursuing the concept of engineered Clostridia to produce anti-cancer drugs (Jennifer et al, 2006). The strictly anaerobic clostridia, on the other hand, have been shown to selectively colonise in solid tumours when delivered systemically and has resulted in high percentage of "cures" of experimental tumours. A phase I clinical trial combining spores of a non toxic strain (C. novyi-NT) with an antimicrotubuli agent has been initiated.

The recombinant DNA technology reignited the field, enabling genetic improvement of Clostridia’s innate oncolytic capability. It provides a possible alternative to overcome the hitch of using wild type strains Anaerobic bacteria, such as Clostridia have now been convincingly shown to selectively colonise and regerminate in the hypoxic/necrotic regions of solid tumours and can be delivered systemically. Furthermore, existing plasmid-based gene modification strategy harbours several safety concerns regarding possible horizontal plasmid transfer and spread of plasmid-associated antibiotic resistant genes.

IV. Current approaches for immunotherapy of cancer

A. Overview

The aim of cancer immunotherapy is to activate patient’s immune system to eradiate tumour cells. It was expected that when appropriately primed, the activated host immune cells, especially tumour antigen specific CD4+ and CD8+ T cells, can specifically kill tumour cells.

Tumour antigens are usually self antigens, both central and peripheral tolerance apply to tumour antigens. Central tolerance occurs in the thymus, T cells with strong self reactivity are eliminated. Peripheral tolerance make tumour specific T cells anergy or suppressive. Cancer vaccine will activate T cells purged of strong activity and influenced by different peripheral tolerance mechanisms. Different approaches have been employed to overcome the tolerance, in order to achieve better T cell responses, including immunization with different routs and with different adjuvant, providing co-stimulating signals while inhibiting signals such as CTLA-4. Neutralizing IL-10 at the same time of immunization has been show to generate better CTL response in antigen experienced host, which is important for cancer immunotherapy; as patients with cancer are tumour antigens experienced.

B. Combining immunostimulation with gene-silencing by siRNA

The innate immune system recognizes pathogens by means of germ line-encoded pattern recognition receptors (PRRs) (Gro F, 2006). A subfamily of PRRs is the Toll-like receptors (TLRs), which is important for initiation of an immune response. siRNAs can activate innate immunity through the activation of Toll-like receptor (Sioud et al, 2007). These findings suggest potential prophylactic and therapeutic use of immunostimulatory siRNAs as adjuvant. In addition, to immune stimulation,

gene-silencing through RNAi is another potency of immunostimulatory siRNAs. RNAi is a widely conserved post-transcriptional gene-silencing mechanism where double-stranded (ds) RNAs trigger the degradation of homologous mRNA sequences and certain siRNA sequences can activate immune cells to secrete proinflammatory cytokines and type I interferons in immune cells. As a consequence of these findings any therapeutic siRNA should be tested in human blood cells prior to use in (Gelman, 2003). However, if we view the activation of innate immunity by siRNAs as beneficial for cancer therapy and infectious diseases, then immuostimulatory siRNAs could emerge as useful agents to knockdown gene expression and activate innate and adaptive immunity against tumour cells. This observation prompted us to design bifunctional siRNAs, which combine gene-silencing and immunostimulation in one single siRNA molecule (Gro F, 2006).

C. Development of strategies to promote effector cell recruitment into tumour

One strategy is to promote effector cell recruitment into metastases when it fails spontaneously (Shakhar, 2003). Intratumoural introduction of chemokines through the use of viral vectors would serve as a proof of concept. Transduction of tumour cells to express specific chemokines has shown benefit in some experimental murine models. Similarly, introduction of the TNF superfamily member LIGHT (homologous to lymphotoxins, inducible expression, competes with HSV glycoprotein D for HVEM, a receptor expressed on T lymphocytes) has been expressed at tumour sites with dramatic results (Kunz M et al, 1999). However, direct intratumoural injection of recombinant viral vectors will only serve as a proof of concept, and development of agents that can be delivered systemically yet target tumour metastases would have to be pursued for practical application.

D. Modulating tumour cell biology to alter the tumour microenvironment

Once the oncogenic signals present in tumour cells that determine the nature of the tumour microenvironment are defined, then it should be possible to target those pathways directly to eliminate the underlying basis for immunosuppression at tumour sites. For example, Stat can drive the expression of vascular endothelial growth factor (VEGF) (Burdelya et al, 2005), which in addition to promoting neoangiogenesis has been reported to be inhibitory for dendritic cell generation in vivo (Della et al, 2005). The interface between tumour biology and the creation of the immunosuppressive tumour microenvironment is an area ripe for additional research.

Another strategy in the immunotherapy of tumours is the use of mRNA-encoding tumour antigens to induce T- and B-cell immunity to the encoded antigens. In vivo application of mRNA induced cytotoxic T-cell activity and specific antibodies in mice. Furthermore, human DCs transfected ex vivo with mRNA induced an antigen-specific immune response both in vitro to a viral antigen and in vivo to a tumour-associated antigen in patients with cancer.

Current efforts in cancer immune therapy and bacteria therapy are largely aimed at stimulating anti-tumour immune responses by using various tumour antigens and adjuvants. The involvement of TLR-activated pathways in immune response is supported by the induction of DC maturation and secretion of various cytokines (Palucka et al, 2007), leading to the induction of innate and adaptive immunity.

E. Targeting cancer stem/progenitor cells for anticancer therapy

The cancer recurrence phenomenon has been associated with the accumulating genetic or epigenic alterations in cancer cells which may contribute to their uncontrolled growth, survival and invasion as well as their intrinsic or acquired resistance to clinical treatments (Lowenberg et al, 2003; Mimeault et al, 2005). Recent investigations have revealed that the most aggressive cancers may originate from the malignant transformation of embryonic or adult stem/progenitor cells into cancer progenitor cells (Mimeault, 2006). The cancer progenitor cells can provide critical functions in cancer initiation and progression into metastatic and recurrent disease states. Numerous investigations have provided evidence that the genetic and/or epigenic alterations occurring in the multi-potent tissue-specific adult stem cells, the most cancers may arise from the malignant transformation of multi-potent tissue-specific adult stem cells and/or their early progenitors into cancer progenitor cells, the accumulation of different genetic and/or epigenic alterations in cancer progenitor cells during cancer progression also seems to be associated with the occurrence of highly aggressive cancer subtypes. The functional properties of cancer progenitor cells may be influenced through external signals mediated by other further differentiated cancer cells and host stromal cells including activated fibroblasts and infiltrating immune cells, such as macrophages and endothelial cells (Kopp et al, 2006).

Among the diverse growth factors, chemokines and angiogenic substances released by stromal cells (Kopp, 2006). All these soluble factors can influence, of autocrine or paracrine manner, the tumour cell behaviour and neovascularization process during cancer progression. The intrinsic or acquired resistance of poorly differentiated and tumourigenic cancer progenitor cells to current clinical therapies may lead to their persistence in primary and secondary neoplasms after treatments, and thereby contribute to cancer recurrence (Mimeault, 2007; de Jonge-Peeters et al, 2007). The cancer stem/progenitor cell model of carcinogenesis may also explain the differences of response of distinct cancer subtypes to current therapies as well as the dormancy phenomenon and disease relapse, which may be associated with a higher resistance of cancer progenitor cells to conventional therapies under specific conditions prevalent in primary and/or secondary neoplasms relative to their further differentiated progeny

(Mimeault, 2007). Based on these observations, the new cancer therapeutic strategies should be based on targeting of different oncogenic cascades activated in tumourigenic cancer progenitor cells, and which must now be considered for improving the current therapeutic treatments. The molecular targeting of tumourigenic cancer progenitor cells must be considered for improving the efficacy of the current cancer therapies.

F. Gene-based tumour immunization

For any gene therapy application including genetic immunization, the goal is to deliver genes into therapeutically-relevant cells while avoiding other cells that cannot contribute to immunization or therapeutic effects. While this is the goal, particularly for in vivo gene therapy, current gene delivery vectors cannot specifically deliver genes to the cells we want and frequently deliver genes into non-target tissues reducing therapy and increasing dangerous side effects.

Generally, the level of gene transfer into tumour cells and immune effector cells determined the level of immunogenetics, they have been shown to be limited, and this has been thought to account for the poor results obtained by cancer gene immunotherapy. Therefore, vector design is one of the most critical areas for future research (Logan et al, 2002). Gene delivery vectors thus are required fall into three areas: 1) identification of cell-targeting ligands using random peptide-presenting phage libraries; 2) engineering viral and non-viral gene delivery vectors to accept cell-targeting ligands; and 3) developing effective methods to image gene and vector delivery in vivo to determine the efficacy of targeted vectors in the complex tumour environment. The different vector systems can have strengths or weaknesses, depending on their use. For ex vivo gene delivery and clinical use in cancer protocols, design of optimized transduction protocols and development of improved vectors, exhibiting improved gene transfer efficiency and stability for large-scale production, have just begun to be evaluated. Nonviral gene delivery systems are cost- and time-effective and large-scale manufacturing of clinical-grade plasmid vectors is logistically simple. The major disadvantages are the low transfection efficiency and the transient expression in target cells. As already mentioned earlier, one of the attractive features of immunological gene therapy approaches is that they capitalize on the ability to amplify the outcome of the gene transfer ('genetic immunopotentiation'). Consequently, high efficiency gene transfer may not be an essential requirement in these protocols. Given this problem, we are interested in developing gene delivery with recombinant engineer bacteria vectors that can be tuned to target specific cells in vivo for gene therapy and immunization applications. As recombinant engineer bacteria are so far the best characterized bacteria vectors, they are most frequently used vectors for immuno-gene therapy of cancer.

Immunogene therapies have the theoretical advantage of inducing a systemic anti-tumour response associated with immunologic memory. Such a response potentially allows for treatment of disseminated disease and a prolonged anti-tumour effect that persists beyond the immediate treatment period. Immunogene therapy strategies involve both ex vivo and in vivo approaches (Glick et al, 2006). Increasing the capacity of the immune system to mediate tumour regression has been a major goal for tumour immunologists. Progress towards tumour vaccines has been recently made by the molecular identification of novel tumour-associated antigens (TAA) and by a better understanding of cellular signals required for efficient T cell activation (Pule et al, 2002). Cancer vaccination is of therapeutic rather than prophylactic nature, involving attempts to activate immune responses against TAA to which the immune system has already been exposed. To date, advances in gene delivery technology have led to the development of immuno-gene therapy strategies to augment host-immune responses to tumours. These approaches include (1) the use of tumour cells genetically modified with genes encoding costimulatory ligands, cytokines or HLA molecules to enhance their immunogenicity and (2) the genetic modification of immune-competent cells with TAA in order to enhance their anti-tumour response.

Despite the continuous increase in clinical gene therapy protocols for immunotherapy of cancer, many aspects of gene transfer are still far from ideal. A basic requirement, not yet adequately and routinely fulfilled, is to introduce the gene of interest with sufficient efficiency into the target cells in order to achieve therapeutic benefit in cancer patients.

G. Breakdown of immune tolerance to tumours

The current rationale lies in the local recruitment of inflammatory cells that can destroy a fraction of the tumour cells directly or indirectly, thereby releasing tumour antigens. These antigens can be taken up in the form of peptides, proteins or apoptotic bodies by professional antigenpresenting cells (APC) by a process known as cross-priming (i.e. indirect presentation of tumour antigens to the immune system by a host-derived APC), that travel to the draining lymph nodes where they will activate naive antigen-specific T cells and initiate a primary cellular immune response. The new approach enlists the help of the immune system to target and kill tumour blood vessel cells, through an unprecedented recruitment of the immune system; they were able to generate a strong anti-tumour effect by targeting the central component of what tumours need most-a blood supply (Niethammer et al, 2002).

According to the classical paradigm in tumour immunology, immune responses are believed to follow a model of discrimination between self and non-self. Consequently, tumours should be considered as non-self, like viruses or bacteria. Therefore, an important task of the immune system is to search for and destroy tumour cells as they arise, in concordance with the original proposals of Burnet's immunological surveillance hypothesis.

However, the limited successes of cancer immunotherapy approaches based on these concepts, prompted a revision of tumour immunology (Luis et al, 2005). Ultimately, it appears that the immune response at the T cell level is based on the presence of the appropriate costimulatory molecules on APC that promote T cell activation. DCs (DC) form a complex network of antigen-capturing and -presenting cells (APC) defined by morphological, phenotypical and functional criteria which distinguish them from monocytes and macrophages (Elke et al, 2002).

Immunity against cancer is necessary if gene transfer is going to be applied in a clinically relevant way. Instead of exploiting the increasing knowledge on cytokines and their plethora of actions in the immune response, immunology may provide a more fundamental mechanism to explain the immunological unresponsiveness to cancer than the classical self/non-self paradigm. At a later stage, we will focus on a new gene-based tumour immunization that seems to fit within this conceptual framework.

H. Stimulation to illicit an active immunoresponse in a solid tumour environment

Van Pel and Boon (1982) demonstrated that a protective immune response could be generated against a 'non-immunogenic' murine tumour, providing the first experimental evidence that the lack of tumour immunity was not due to the absence of TAA but rather to the inability to stimulate the immune system. Factors that can explain the failure of the immune system in tumour-bearing hosts are numerous, and it is not clear which of them are critical in the clinical context. We all know that tumour cells are poor stimulators of immune responses and capable of inducing immune tolerance. Alternatively, it may well be that the lack of costimulatory molecules (e.g. CD80, CD86) on the surface of tumour cells accounts for the immune tolerance which keeps the tumour from being rejected. Deficiency of the immune system could be responsible for the lack of immunity and induction of T cell tolerance (von Euler et al, 2008). In this case; the tumour actively suppresses host antigen presentation and immune effect or functions by expression of a variety of local inhibitory molecules, such as VEGF and IL-10, especially when large tumour burdens are involved.

Antigen-specific cytotoxic cells that do specifically recognize tumour cells can be generated by cell cloning techniques ex vivo or can be genetically engineered by the stable transfection of a TCR that specifically recognizes a certain MHC-tumour antigen complex (Keith et al, 2002). This has been made possible by the use of defined tumour antigens to stimulate lymphocytes in vitro, and the ability to clone lymphocytes derived from a single, antigen-specific T cell (Pule et al, 2002). Adoptive transfer of clonally expanded lymphocytes to lymphopenic hosts after nonmyeloablative conditioning chemotherapy has resulted in cell proliferation and persistent clonal repopulation correlated with tumour regressions in patients with melanoma (Keith et al, 2002). Ex vivo–expanded clonal populations of tumour antigen–specific lymphocytes can be derived from a natural or genetically engineered initiating cell. Moreover, the TCR of cytotoxic T cells can be substituted with an immunoglobulin-like surface molecule, which allows the binding to tumour-specific surface molecules not presented by MHC molecules (Keith et al, 2002). These more elaborate forms of adoptive transfer of killer cells are being studied in ongoing clinical trials. A second approach in preclinical development involves genetic modification of DCs with the gene for interleukin-7 (IL-7). IL-7 stimulates cytotoxic T-lymphocyte responses and down-regulates tumour production of the immunosuppressive growth factor, TGF-β.

V. Cancer vaccine

A. Overview

In the past two decades, adoptive immunotherapy, based on tumour-infiltrating lymphocytes or lymphokine-activated killer cells, has been used in clinical trials (Rosenberg et al, 1986; Rosenberg et al, 1987). These early results gave first evidence that the manipulation of the immune system represents a promising tool in cancer immunotherapy. The main rationale of genetic immunopotentiation protocols is the possibility of enlisting the immune system for a potentially vast amplification of gene therapy, thereby enhancing therapeutic benefit. The recognition that most tumours encode TAA and are capable of inducing protective immunity in preclinical models has reinvigorated the field of cancer immunotherapy (Pule et al, 2002). It has been hypothesized that the immune system of tumour patients, characterized by tolerance, can be modified to mount an immunological response against the tumour and thus facilitate tumour rejection. This 'cancer vaccination' is to be accomplished through exposure of TAA in a more favourable context to the immune system (Christian et al, 2006). Despite ongoing efforts to define and characterize TAA and, more importantly, clinically relevant TAA, little is known about TAA for the majority of human cancers and the largest part of clinical experience with tumour vaccines has been obtained in melanoma patients. Therefore, most cancer vaccines, to date, use tumour cells as a source of TAA. The molecular identification of antigens expressed by tumour cells that can be recognized by specific $CD8^+$ cytotoxic T lymphocytes (CTLs) has provided a means by which to explore anti-tumour T-cell parameters in patients and also to develop antigen-specific immunotherapies.

B. Current vaccines

1. Antigen Presentation to the Immune System

The immune system responds to intracellular events in target cells by the recognition of intracellularly derived protein fragments presented on the cell surface by major histocompatibility complex (MHC) molecules. Circulating T lymphocytes can potentially engage these peptide-MHC complexes through their T-cell receptors (TCR). This

mechanism allows the immune system to differentiate abnormal intracellular processes from normally functioning cells expressing so-called self proteins. The key steps in the generation of an immune response to cancer cells include loading of tumour antigens onto antigen-present cells in vitro or in vivo (**Figure 1**).

2. Intratumoral bacillus Calmette-Guérin (BCG)

This strategy may be one of the earliest forms of cellular immunotherapy tested by the Intratumoral injection of the BCG in cancer (Mathe et al, 1973). The immunologic basis is that BCG generates an inflammatory process ideal for the attraction of APCs, which pick up tumour antigens released by the tumour cells, damaged by the bacterial infection and cross-present them in a so-called danger environment. This form of treatment generates occasional antitumor immune responses.

3. Intratumoral HLA-B7

The intratumoral injection of BCG, the recognition of a powerful alloantigen by cells with NK activity allows the recruitment of APCs, among other inflammatory cells, which will pick up tumour antigens released by the HLA-B7–transfected cells and cross-present them to cytotoxic effector cells. These tumours antigen-specific $CD8^+$ CTLs would then be permitted to attack other tumour cells without the requirement of the presence of the alloantigen HLA-B7 on tumour cells.

4. Whole-cell tumour vaccines

Whole-cell autologous tumour vaccines are personalized vaccines, and it can be assumed that they contain the relevant tumour antigens; however, the logistic drawback is that it is difficult to obtain and individually prepare vaccines for each patient. To avoid this problem, other tumour cell vaccines have been formulated as lysates of allogeneic laboratory cell lines containing shared tumour antigens (Sondak et al, 2002).

5. Naked DNA and gene-modified tumour vaccines

Intramuscular injection of naked DNA sequences results in gene expression and the generation of immune responses (Wolff et al, 1990; Kumar et al, 1996). These DNA plasmids, which consist of an antigen gene regulated by a promoter with constitutive activity can be conjugated with gold particles and propelled into the skin using a helium gas gene gun. The protein antigen produced by the target cells is taken up by host APCs, processed, and cross-presented to the immune system in the draining lymph nodes.

Gene-modified tumour vaccines have been tested in clinical trials for many years, the paracrine expression of cytokines such as IL-2 or IFNγ, would allow the tumour cell to provide all of the signals for direct cytotoxic T cell activation, bypassing the need for host APCs and $CD4^+$ T lymphocyte assist (Fearon et al, 1990). However, comparison of the antitumor capacity of gene-modified tumour vaccines in preclinical models was surprising in that the introduction of GM-CSF into tumour cells produced the most active vaccine (Dranoff et al, 1993). Bone marrow chimeras were used to show that the GM-CSF gene-modified tumour vaccines attracted host APCs, which picked up tumour antigens and cross-presented them to the host immune system (Huang et al, 1994).

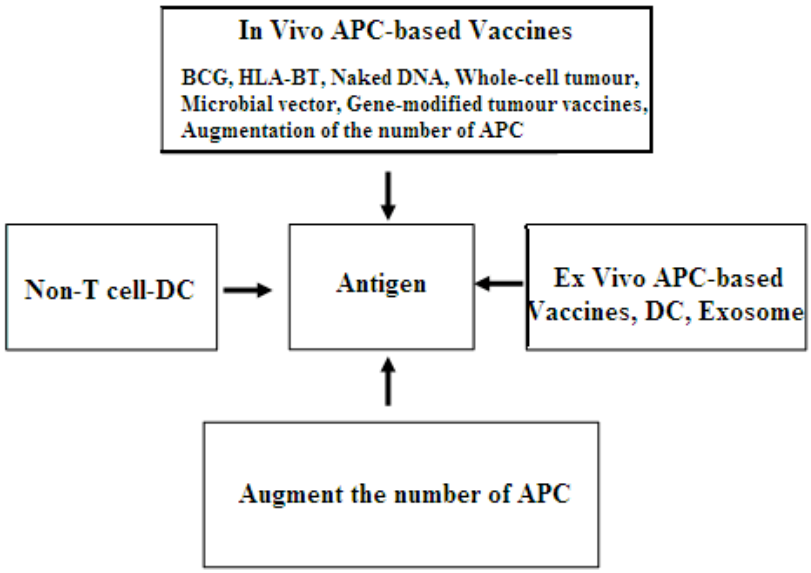

Figure1: Cross-presentation of tumour antigens derived from cancer vaccines.
Several immunologic manipulations lead to a common pathway of cross presentation of proteins derived from tumour antigens. **a**) in vivo APC-Based Vaccines; **b**) ex Vivo APC-Based Vaccines; **c**) augment the number of APC; **d**) non-T cell-DC. These host antigen-presenting cells (APCs), the most powerful of which are the DCs, circulate through the afferent lymphatic vessels to the T-cell areas of lymph nodes. There they cross-present the tumour antigen to T lymphocytes.

6. Microbe-based vaccines

A variety of microbiology vectors have been adapted to cancer immunotherapy. Tumour antigen DNA sequences can be inserted into attenuated pox viruses that are unable to replicate in mammalian hosts or tumour antigen gene segments have been introduced into bacteria such as Salmonella and Listeria, resulting in protective immunity in animal models (Huang et al, 1994). Other vectors include recombinant replication-incompetent viral vectors (adenovirus, retrovirus, lentivirus), which are modified viruses that have been specifically mutated to be incapable of self-replication into infectious progeny virions after infection of a single target cell, but that efficiently express the foreign gene inserted in the vector. This form of genetic immunization has also resulted in weak immunologic responses in humans (Rosenberg et al, 1998), enhancing the immune potency of viral vector. Immunization can be achieved by the coexpression of cytokines or costimulatory molecules in the viral vector because these viral vectors usually have a large capacity to carry and express multiple genes (Rosenberg et al, 1998). Several anaerobic bacteria vectors are testing in lab now. Advantages may include the ability to use the oral route for immunization and the strong inflammatory milieu created by bacterial products, leading to the attraction of APCs, and a preferential Th1 cytokine polarizing pattern stimulated by certain bacteria such as Listeria.

7. The prime-boost strategy

The sequential administration of naked DNA and a viral vector has resulted in synergistic immune activation; it is a potent method of generating immune responses to tumour antigens in what is now known as the prime-boost strategy. The initial injection of a plasmid allows the activation of infrequent T cells without other immune cells competing for the antigen because the naked DNA has a limited inflammatory potential. After a rest period, these antigen-specific high-avidity lymphocytes are boosted by the re-exposure to the same antigen, now in a more inflammatory milieu generated by the highly immunogenic viral proteins from the recombinant viral vector. Preclinical murine and primate models have shown that this heterologous prime-boost regimen induces 10- to 100-fold higher frequencies of T cells than do naked DNA or recombinant viral vectors alone (Ramshaw et al, 2000). A modification of this strategy is the sequential administration of two different viral vectors carrying the same tumour antigen gene, which bypasses the limitation of the development of neutralizing antibodies to the viral backbone by boosting with a different vector without shared viral epitopes (Mincheff et al, 2000; Marshall et al, 2000). These strategies, which avoid the need of cell culture common to the majority of highly immunologically active vaccine strategies, are rapidly undergoing clinical testing for infectious disease and cancer.

8. Augmentation of the number of APCs

As can be noted by the mechanism of action of most of the prior immunologic maneuvers, the common pathway of anticancer immune activation is the recruitment and activation of host APCs to cross-present tumour antigens to effector $CD8^+$ cytotoxic T cells (**Figure1**). Cytokines such as GM-CSF have been used as vaccine adjuvants with the hope of attracting and activating DCs locally at the site of vaccination. Other strategies are aimed at systemically expanding the dendritic cell pool in the hosts, which may be achieved by the administration of cytokines such as the combination of GM-CSF and IL-4 (Roth et al, 2000). In retrospective studies of tumour biopsies, a greater number of APCs infiltrating the cancer have been correlated with improvements in survival of patients (Lotze, 1997). This increase in the availability of intratumoral APCs may allow more efficient cross-presentation of tumour antigens.

C. Ex vivo APC-based vaccines

1. DCs and exosomes

The crucial role of DCs was discovered for the induction of primary T-cell–dependent immune responses. DCs are now considered to be the best adjuvant for antitumor immunity. Different antigen loading procedures have been used for dendritic cell antigen presentation. For well-characterized antigens, synthetic HLA-binding peptide epitopes or the complete DNA sequence in a viral vector can be used to load the dendritic cell vaccines. DCs pulsed with peptide epitopes and genetically-modified with recombinant viral or bacteria vectors are conceptually similar to the vaccination with peptides in immunologic adjuvants or the direct administration of recombinant viruses, respectively, in which the DCs should be perceived as powerful immunologic adjuvants for the tumour antigen. Also, DCs can be loaded with defined antigens to take advantage of antigen uptake surface receptors, such as FC receptors to take up immune complexes carrying a tumour antigen (Rafiq et al, 2002).

The nanometer vesicles derived from late endosomes are released differentiated in vitro by DCs , which contain most of the appropriate molecules to adequately present MHC-antigen complexes to the immune system (Wolfers et al, 2001; Zitvogel et al, 1998). These exosomes can be isolated by filtration of dendritic cell culture media and then loaded with custom antigens. Their use alone as vaccines or as vehicles to transfer back preassembled MHC-peptide complexes to DCs is under clinical investigation

2. Non–T-cell–directed cancer vaccines

Monoclonal antibodies to surface receptors, such as trastuzumab or rituximab, have complex mechanisms of action leading to effective tumour regressions. One such mechanism is the stimulation of antibody-dependent cell-mediated cytotoxicity. This immune-based effect, together with the recognized ability of immune complexes to allow antigen cross-presentation in DCs (Clynes et al, 2000), may contribute to their antitumour effects by a coordinated humoral and cellular response. Several other cancer vaccines are in different phases of clinical testing. Most of

these strategies rely on the activation of humoral (antibody) responses to a peptide or nonpeptide antigen. Resultant tumour cell damage and cross-presentation of antigen by host APCs may allow the transfer of the immunologic stimulus to cellular immune responses.

Advances in the understanding of the mechanisms of action of cellular antitumour immune responses have allowed the development of new generations of cancer vaccines, in which the key step is the recognition of the need for professional APCs to cross-present the antigen to the host immune system. The most immunologically active vaccines usually require costly and laborious ex vivo cellular cultures, whereas the cell-free vaccines that can be directly administered from an easily stored and transported vial are usually less immunologically active but more suitable for widespread clinical testing. New advances in the formulation of cancer vaccines brought by a more precise knowledge of the requirements for the generation of cellular immune responses to tumour antigens, together with the current ability to closely monitor cellular immune responses, will likely provide powerful, nonindividualized, cell-free vaccines in the near future.

VI. Combined multi-modality therapy: immunization with anaerobic bacteria therapy for tumour

Immunotherapy strategies for cancer gene therapy utilize gene transfer to facilitate a dormant host immune response directed against the tumour. Evasion of autologous host cellular immunity is a common feature of tumour cell neoantigens. Tumour cells are poor antigen presenting cells. 'Cancer vaccine' strategies are based on optimization of the context in which tumour antigens or tissue-specific antigens are presented to the host immune system (Sobol et al, 1995). Utilizing gene therapy to optimize tumourantigen presentation is through the targeted expression of cytokines in tumour cells. Targeted paracrine expression eliminates the toxicities associated with systemic cytokine administration. The transduced cytokines result in a combination of improved tumour cell vaccine antigen presentation, and activation of APCs, both essential for effective priming of the cellular immune response.

The vector-induced inflammatory/immune response functions as an adjuvant to the transduced antigen, resulting in local release of cytokines and influx of APCs to the vaccine site. The immunotherapy of cancer is now being assessed in the clinics. An immune response has a potentially long-term clinical impact on the course of the disease by stabilising the condition and thus prolonging survival rather than by performing massive tumour elimination, those with minor tumour burden or patients who have had their tumour surgically removed but who have a high risk of relapse. In these categories of patients, disease stabilisation, frequency of relapse, time-span to relapse and length of survival are the most rational parameters for evaluating cancer immunization effectiveness. Even if optimal gene delivery is achieved, the success of gene therapy, like conventional therapy, may be impeded by tumour cell resistance and intratumoural cell heterogeneity. The use of combined treatment modalities provides a rational paradigm to improve upon the clinical efficacy of cancer gene therapy (Klencke et al, 2002). Within the modality of gene therapy itself, multiple therapies may be combined in an attempt to benefit from additive or synergistic efficacy. Multi-gene therapy approaches already under evaluation include the transduction of dual immunostimulatory molecules for immunotherapy, and anaerobic bacteria therapy (**Figure 2**).

A major limitation in the use of gene therapy in solid tumours in vivo is the diffusion-limited tissue penetration into the target tissue. The ability of immunotherapy and anaerobic bacteria therapy has been observed in vitro and in vivo. The effects we observed in animals are contingent on both bacteriolysis and immunity. There are three reasons to believe that systemic injection of Clostridium. Novyi-NT (C. novyi-NT) into humans would lead to bacteriolysis of tumours. First, C. novyi-NT germinates within the tumours of all three species tested (rabbits, rats, and mice), whether the tumours are s.c., intramuscular, or intrahepatic. Second, C. novyi-NT can germinate within human tumour xenografts in the nude mouse host (although complete regressions and cures are not generally observed as there is minimal T cell-mediated immunity). And third, there are many case reports of C. novyi germination and gangrene developing in penetrating wounds or after illicit drug injection. These reports demonstrate that the parental strain of C. novyi, differing from C. novyi-NT only in that the latter is devoid of the lethal α-toxin gene, can proliferate within hypoxic regions in humans.

C. novyi-NT infection of cancers in humans will induce tumour immunity is more difficult to predict (Dang et al, 2004). There are many studies indicating that human tumours are immunogenic, as assessed by the presence of specific antibodies or reactive T cells in untreated patients. Furthermore, it has been shown that stronger immune responses can be elicited through the administration of various vaccines in several clinical trials. But there are also many studies indicating that human tumour cells can protect themselves against potential immune responses through a variety of direct and indirect mechanisms.

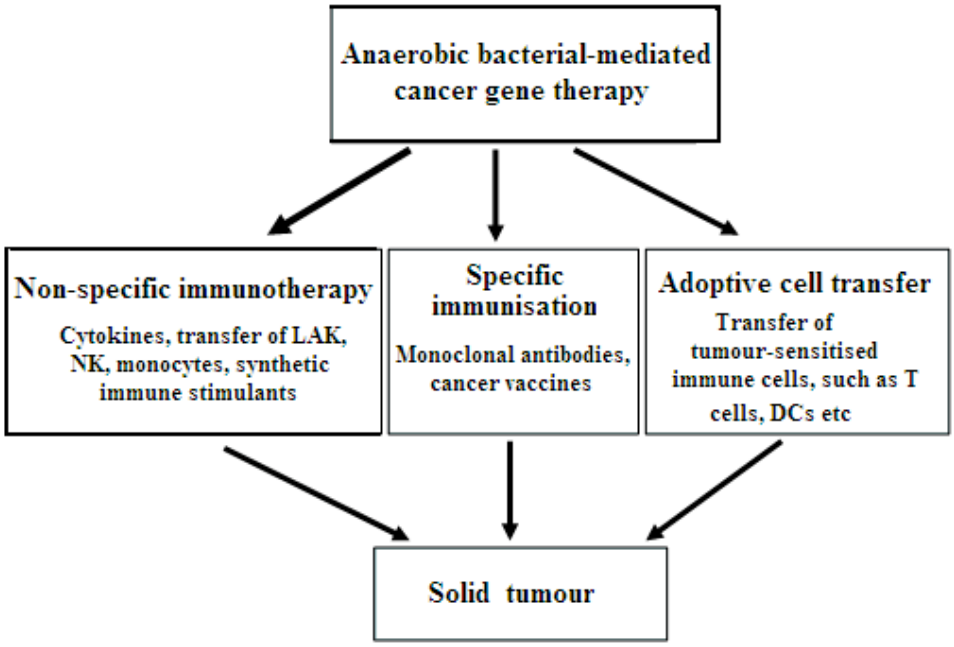

Figure 2: Anaerobic bacteria-mediated immunologic therapy for solid tumour
Anaerobic bacteria therapy has been observed these effects in treatment of solid tumour: **a**) nonspecific immunologic therapy which the characterization of cytokines produced by immune system cells and their production by genetic recombinant techniques, such as IL-2 and IFN, the significant toxicity of high-dose systemic cytokine therapy is the major drawback; **b**) specific immunisation represent which allow the stimulation of an immune response while avoiding the high toxicity of systemic administration of recombinant anaerobic bacteria vectors and gene modification of tumour cells, which allows an initial direct cytotoxic effect on the cancer cell by antibody dependent cellular cytotoxicity, thereby releasing tumour antigens; **c**) the adoptive transfer of immune effector cells from the immune system, T cell, DCs pulsed with genetically-modified with recombinant anaerobic bacteria vectors are conceptually similar to the vaccination with peptides in immunologic adjuvant.

As similar observations, both with respect to the potential of tumours to elicit an immune response and their ability to evade such responses, have been recorded in animals, there is reason to hope that the immune therapeutic effects stimulated by C. novyi-NT germination might be obtainable in carefully selected patients.

In experimental setting, the strictly anaerobic Clostridia have demonstrated several advantages over others as clostridial spores specifically colonise and germinate into vegetative cells in the hypoxic regions of solid tumours, causing tumour lysis and destruction. Early trials in the 70's of non pathogenic strains in human had shown plausible safety (Carey et al, 1976).

VII. Conclusions

Current innovative approaches for cancer therapy hold significant potentials for effective cancer management; bacteria therapies and immunotherapies will probably be the most promising, especially when genetic manipulation of bacteria to improve its potential have applied. Recent understanding of tumour microenvironment, detailed characterization of tumour antigens and the increased revealing of the immunological pathways involved in tumour immunity have paved the way for the design of gene-immune therapies (Ribas et al, 2000). To this end, three cellular sources can be envisaged for genetic modification: tumour cells, effector T cells and DCs. However, before ex vivo immuno-gene therapy can become a realistic treatment modality for cancer, several barriers have yet to be overcome. First, improved (bacteria) vectors should lead to higher gene delivery rates and transgene expression. Therefore, carefully designed clinical studies are necessary to assess gene transfer efficiency, safety and toxicity, and eventually to establish the clinical efficacy of the tumour immunization. With regard to gene-modified tumour cells, another major issue still unsolved at the clinical level is to determine what is the best cytokine the tumour cells to release in order to recruit the immune system. Second, it will be imperative to break down the immunological tolerance against the tumour through reversal of T cell ignorance, anergy or tumour-induced immunosuppression in order to achieve a therapeutic outcome. Use of DCs, whether gene-modified or not, in the context of danger signals could provide a means to initiate a cellular immune response against the tumour. An additional general feature to be considered when designing immuno-gene therapy of cancer is the complex redundancy of the immune system. Its effectiveness in protecting the body from harmful infections demands a sophisticated network to control the pathways of activation and termination of an immune response, as well as maintenance of life-long tolerance. This suggests that a combination of multiple strategies,

gene-based or not, acting at different levels may be advantageous to boost the immune system against the tumour. Moreover, it is believed that the breakdown of tolerance to tumours will require, in addition to the strategies discussed in this review, complementary strategies that specifically counteract the active tumour-induced immunosuppression.

VIII. Future directions

The challenges facing the implementation of successful gene therapeutic strategies will be better understood as the early clinical trials for cancer gene therapy begin to return more results. Vector development with increased transgene size capacity, optimized immunogenic properties, and improved gene transfer efficiency and targeting will facilitate the next generation of gene therapy strategies (Kanai et al, 1998). The burgeoning field of genomics provides an exciting new resource for the design of tumour-specific gene therapy strategies. Harnessing these tumour gene products and others for use as immunization offers exciting prospects for a whole new class of cancer gene therapy strategies. As the diversity of molecular lesions underlying tumourigenesis is better characterized, new targets for corrective and cytoreductive approaches will emerge. Effective anticancer gene therapy may ultimately require individualized molecular profiles. Solid tumours meet their demands for nascent blood vessels and increased glycolysis, to combat hypoxia, by activating multiple genes involved in angiogenesis and glucose metabolism. Hypoxia inducible factor-1(HIF-1) is a constitutively expressed basic helix-loop-helix transcription factor, formed by the assembly of HIF-1alpha and HIF-1beta, which is stabilized in response to hypoxia, and rapidly degraded under normoxic conditions (Kanai et al, 1998). It activates the transcription of genes important for maintaining oxygen homeostasis but failed to stimulate systemic T-cell-mediated antitumour immunity, and synergized with B7-1-mediated immunotherapy. This approach holds promise to form the foundation for the transition between the traditional anticancer therapies and the molecular antineoplastic gene therapy of the future. Other approaches are to develop new gene therapy vectors whose expression is selectively activated by hypoxia (Rosenberg et al, 1998). As VEGF is upregulated by hypoxia, such regulatory mechanisms would enable us to achieve hypoxia-inducible expression of therapeutic genes. The unique pathophysiology of solid tumours presents a huge problem for the conventional therapies. Thus, the outcomes of current therapies are so far disappointing. Several new approaches aiming at developing effective treatments are on the horizon. These include a variety of bacteria-based therapy systems. Amongst all these, anaerobic bacteria vector-mediated cancer therapy is most promising and expected to generate new data and new protocols for cancer gene therapy.

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References

Agrawal N, Bettegowda C, Cheong I, et al (**2004**). Bacteriolytic therapy can generate a potent immune response against experimental tumors. **Proc Natl Acad Sci U S A** 101, 15172-7.

Akasaki Y, Liu G, Matundan HH, et al (**2006**). A peroxisome proliferator-activated receptor-gamma agonist, troglitazone, facilitates caspase-8 and -9 activities by increasing the enzymatic activity of protein-tyrosine phosphatase-1B on human glioma cells. **J Biol Chem** 281, 6165-74.

Aznavoorian S, Stracke ML, Krutzsch H, Schiffmann E, Liotta LA (**1990**). Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. **J Cell Biol**; 110**,** 1427-38.

Akbulut L, Zhang Y, Deisseroth A (**2003**). Cytotoxic effect of replication-competent adenoviral vectors carrying L-plastin promoter regulated E1A and cytosine deaminase genes in cancers of the breast, ovary and colon, **Cancer Gene Ther** 10, 388–95.

Bubenik J, Vonka V (**2003**). MHC class I status of tumours and design of immunotherapeutic strategies. **Immunol Lett** 90**,** 177-8.

Biswas S, Guix M, Rinehart C, et al (**2007**). Inhibition of TGF-beta with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. **J Clin Invest** 117**,** 1305-13.

Bos R vDP, van der Groep P, Shvarts A, Greijer AE, van der Wall E (**2004**). Expression of hypoxia-inducible factor-1alpha and cell cycle proteins in invasive breast cancer are estrogen receptor related. **Breast Cancer Res** 6: R450-9.

Burdelya L, Kujawski M, Niu G, et al (**2005**). Stat3 activity in melanoma cells affects migration of immune effector cells and nitric oxide-mediated antitumor effects. **J Immunol** 174, 3925-31.

Berzofsky JA, Terabe M (**2008**). NKT cells in tumor immunity: opposing subsets define a new immunoregulatory axis. **J Immunol** 180, 3627-35.

Battaglia M, Gregori S, Bacchetta R, Roncarolo MG (**2006**). Tr1 cells: from discovery to their clinical application. **Semin Immunol** 18, 120-7.

Bonizzi RP (**2007**). Welfare and immune response. **Veterinary Research Communications** 31, 97-102.

Cho W. Contribution of oncoproteomics to cancer biomarker discovery (**2007**). **Mol Cancer** 6, 25-9.

Croker AK, Allan AL (**2008**). Cancer stem cells: implications for the progression and treatment of metastatic disease. **J Cell Mol Med** 12**,** 374-90.

Chen ZS, Robey RW, Belinsky MG, et al (**2003**). Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. **Cancer Res** 63, 4048-54.

Cools N, Ponsaerts P, Van Tendeloo VF, Berneman ZN (**2007**). Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. **J Leukoc Biol** 82, 1365-74.

Caux CD, Schmitt D, Banchereau J (**1992**). GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. **Nature** 360, 258-61.

Clynes RA, Towers TL, Presta LG, et al (**2000**). Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. **Nat Med** 6, 443–6,

Carey RW, Holland JF, Whang HY, Neter E (**1967**), Bryant B: Clostridial oncolysis in man. **Euro J Can** 3, 37-46.

Christian A Petrulio SK-S, Howard L Kaufman (**2006**). The tumour microenvironment and implications for cancer immunotherapy. **Expert Opinion on Biological Therapy** 6, 671-84.

Dong H, Chen L (**2003**). B7-H1 pathway and its role in the evasion of tumor immunity. **J Mol Med** 81**,** 281-7.

Della Porta M, Danova M, Rigolin GM, et al (**2005**). Dendritic cells and vascular endothelial growth factor in colorectal cancer: correlations with clinicobiological findings. **oncology** 68, 276-84.

Dean M, Fojo T, Bates S (**2005**). Tumour stem cells and drug resistance. **Nat Rev Cancer** 5, 275-84

Dranoff G, Jaffee E, Lazenby A, et al (**1993**). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. **Proc Natl Acad Sci U S A** 90, 3539–43.

Dang LH, Bettegowda C, Huso DL, Kinzler KW, Vogelstein B (**2001**). Combination bacteriolytic therapy for the treatment of experimental tumors. **Proc Natl Acad Sci U S A** 98, 15155-60.

Dang LH, Bettegowda C, Agrawal N, et al. (**2004**). Targeting vascular and avascular compartments of tumors with C. novyi-NT and anti-microtubule agents. **Cancer Biol. Ther**. 3, 326-337

de Jonge-Peeters SD, Kuipers F, de Vries EG, Vellenga E (**2007**). ABC transporter expression in hematopoietic stem cells and the role in AML drug resistance. **Crit Rev Oncol Hematol** 62**,** 214-26.

Elke Jäger DJ, Alexander Knuth (**2002**). Clinical cancer vaccine trials. **Current Opinion in immunology** 14, 178-82.

Frey AB (**2006**). Myeloid suppressor cells regulate the adaptive immune response to cancer. **J Clin Invest** 116, 2587-90.

Fearon ER, Pardoll DM, Itaya T, et al (**1990**). Interleukin-2 production by tumour cells bypasses T helper function in the generation of an antitumor response. **Cell** 60, 397–403.

Gro F MS (**2006**). Design of bifunctional siRNAs: Combining immunostimulation and gene-silencing in one single siRNA molecule. **Biochemical and Biophysical Research Communications** 352**,** 642-9.

Ganss R (**2006**). Tumor stroma fosters neovascularization by recruitment of progenitor cells into the tumor bed. **J Cell Mol Med** 10, 857-65.

Glick RP LT, Lin H, Tarlock K, Cohen EP (**2006**). Immunogene therapy as a treatment for malignant brain tumors in young mice. **J Neurosurg** 105, 65-70.

Gelman AE (**2003**). Autoimmunity heats up. **Nat Med** 9, 1465-6.

Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM (**2005**). Expression and function of laminins in the embryonic and mature vasculature. **Physiol Rev** 85,979-1000.

Huang AY, Golumbek P, Ahmadzadeh M, et al (**1994**). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. **Science** 264, 961–5.

Iwadate Y, Namba H, Tagawa M, et al (**1997**). Induction of acquired immunity in rats that have eliminated intracranial gliosarcoma cell by the expression of herpes simplex virus thymidine kinase gene and ganciclovir administration. **Oncology** 54, 329-34

Jennifer A L, Philipp K (**2006**). Recent developments in antibacterial drug discovery: microbe-derived natural products – from collection to the clinic. **Expert Opinion on Investigational Drugs** 15**,** 211-26.

Koido S, Nikrui N, Ohana M, et al (**2005**). Assessment of fusion cells from patient-derived ovarian carcinoma cells and dendritic cells as a vaccine for clinical use. **Gynecol Oncol** 99, 462-71.

Kay A (**2001**). Allergy and allergic diseases. First of two parts. **N Engl J Med** 344, 30-7.

Keith LK BA, David A M, Kathy S, Mary LD (**2002**). Adoptive T-cell therapy for the treatment of solid tumours. **Expert Opinion on Biological Therapy** 2, 55-66.

Klencke B, Matijevic M, Urban RG, et al (**2002**). Encapsulated plasmid DNA treatment for human papillomavirus 16-associated anal dysplasia: A phase I study of ZYC101. **Clin Cancer Res** 8, 1028–37,

Kanai F, Kawakami T, Hamada H, et al (**1998**). Adenovirus-mediated transduction of *Escherichia coli* uracil phosphoribosyltransferase gene sensitizes cancer cells to low concentrations of 5-fluorouracil. **Cancer Res** 58, 1946-51.

Kunz M TA, Goebeler M, Engelhardt E, Bröcker E, Gillitzer R (**1999**). Strong expression of the lymphoattractant C-X-C chemokine Mig is associated with heavy infiltration of T cells in human malignant melanoma. **J Pathol** 189, 552-8.

Kopp HG, Ramos CA, Rafii S (**2006**). Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. **Curr Opin Hematol** 13, 175-81.

Kumar V, Sercarz E (**1996**). Genetic vaccination: The advantages of going naked. **Nat Med** 2, 857–9.

Logan AC, Lutzko C, Kohn DB (**2002**). Advances in lentiviral vector design for gene-modification of hematopoietic stem cells. **Curr Opin Biotechnol** 13, 429-36.

Lotze MT (**1997**). Getting to the source: Dendritic cells as therapeutic reagents for the treatment of patients with cancer. **Ann Surg** 226, 1–5,

Lowenberg B, Griffin JD, Tallman MS (**2003**). Acute myeloid leukemia and acute promyelocytic leukemia. **Hematology Am Soc Hematol Educ Program** 82-101.

Luis SF, Eckhard RP (**2005**). Lung Cancer Immunotherapy. **Clin Med Res** 3, 221-28.

Mahoney BP, Baggett B, Gillies RJ (**2003**). Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro. **Biochem Pharmacol** 66, 1207-18.

Morgan RA, Dudley ME, Wunderlich JR (**2006**). Cancer regression in patients after transfer of genetically engineered lymphocytes. **Science.** 314(5796), 126-9

Marina Mata JCG, David J. Fink (2003). Gene Transfer to the Nervous System: Prospects for Novel Treatments Directed at Diseases of the Aging Nervous System. **The Journals of Gerontology Series A: Biological Sciences and Medical Sciences** 58, M1111-18

Mimeault M, Batra SK (**2006**). Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. **Stem Cells** 24, 2319-45.

Mimeault M, Batra SK (**2007**). Interplay of distinct growth factors during epithelial mesenchymal transition of cancer progenitor cells and molecular targeting as novel cancer therapies. **Ann Oncol** 18**,** 1605-19.

Mathe G, Halle-Pannenko O, Bourut C (**1973**). BCG in cancer immunotherapy: Results obtained with various BCG preparations in a screening study for systemic adjuvants applicable to cancer immunoprophylaxis or immunotherapy. **Natl Cancer Inst Monogr** 39, 107–13.

Martin-Orozco N, Dong C (**2006**). New battle fields for costimulation. **J Exp Med** 203**,** 817-20.

Mimeault M, Brand RE, Sasson AA, Batra SK(**2005**). Recent advances on the molecular mechanisms involved in pancreatic cancer progression and therapies. **Pancreas** 31, 301-16.

Mimeault M, Batra SK (**2006**). Recent advances on multiple tumorigenic cascades involved in prostatic cancer progression and targeting therapies. **Carcinogenesis** 27, 1-22.

Marshall JL, Hoyer RJ, Toomey MA, et al (**2000**). Phase I study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. **J Clin Oncol** 18, 3964–73,

Mincheff M, Tchakarov S, Zoubak S, et al (**2000**). Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: A phase I/II clinical trial. **Eur Urol** 38**,** 208–17.

Mabjeesh N J, Simons J W (**2002**). Gene therapy of prostate cancer: current and future directions. **Endocrine-Related Cancer** 9, 115-39.

Nuyts S VML, Theys J, Landuyt W, Lambin P, Anné J (**2002**). Clostridium spores for tumor-specific drug delivery. **Anticancer Drugs** 13, 115-25.

Niethammer AG, Xiang R, Becker JC, et al (2002). A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. **Nat Med** 8, 1369-75.

Ohtani H (**1998**). Stromal reaction in cancer tissue: pathophysiologic significance of the expression of matrix-degrading enzymes in relation to matrix turnover and immune/inflammatory reactions. **Pathol Int** 48**,**1-9.

Pouysségur J DF, Mazure NM (**2006**). Hypoxia signalling in cancer and approaches to enforce tumour regression. **Nature** 441, 437-43.

Petrulio CA, Kim-Schulze S, Kaufman HL (**2006**). The tumour microenvironment and implications for cancer immunotherapy. **Expert Opin Biol Ther** 6, 671-84.

Pule BC, Heslop HE (**2002**). Genetically engineered T-cells for adoptive immunotherapy. **Curr Opin Mol Ther** ; 4, 467-75.

Philip W, Wong CL, Ian F (**2005**). Tannock Reduction of Intracellular pH as a Strategy to Enhance the pH-Dependent Cytotoxic Effects of Melphalan for Human Breast Cancer Cells **Clinical Cancer Research** 11**,** 3553-7.

Palucka AK, Joseph W F, Jacques B (**2007**). Taming cancer by inducing immunity via dendritic cells. **Immunological Reviews** 220, 129-50.

Roth MD, Gitlitz BJ, Kiertscher SM, et al (**2000**). Granulocyte macrophage colony-stimulating factor and interleukin 4 enhance the number and antigen-presenting activity of circulating CD14+ and CD83+ cells in cancer patients. **Cancer Res** 60, 1934–41.

Rafiq K, Bergtold A, Clynes R (**2002**). Immune complex-mediated antigen presentation induces tumor immunity. **J Clin Invest** 110, 71–79.

Reilly RT, Gottlieb MB, Ercolini AM, et al (**2000**). HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. **Cancer Res** 60, 3569-76.

Rosenberg SA, Zhai Y, Yang JC, et al (**1998**). Immunizing patients with metastatic melanoma using recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. **J Natl Cancer Inst** 90, 1894–1900.

Ramshaw IA, Ramsay AJ (2000). The prime-boost strategy: Exciting prospects for improved vaccination. **Immunol Today** 21**,** 163–5.

Rosenberg SA, Spiess P, Lafreniere R (**1986**). A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. **Science** 233, 1318–1321.

Rosenberg SA, Lotze MT, Muul LM, et al (**1987**). A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. **N Engl J Med** 316**,** 889–97.

Ribas A, Butterfield LH, Economou JS (**2000**). Genetic immunotherapy for cancer. **Oncologist** 5, 87–98.

Shicang Y, Guijun H, Guisheng Q, Yuying L, Guoming W, Ruiling G (**2007**). Efficacy of chemotherapeutic agents under hypoxic conditions in pulmonary adenocarcinoma multidrug resistant cell line. **J Chemother** 19, 203-11.

Suciu-Foca N, Manavalan JS, Cortesini R (**2003**). Generation and function of antigen-specific suppressor and regulatory T cells. **Transpl Immunol** 11, 235-44.

Sallusto FA (**1994**). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. **J Exp Med** 179, 1109-18.

Steinman RM, Turley S, Pierre P, Mellman I (**1999**). Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies. **Hum Immunol** 60,562-7.

Seo N, Hayakawa S, Takigawa M, Tokura Y (**2001**). Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. **Immunology** 103, 449-57.

Sioud M FG, Cekaite L (**2007**). Suppression of immunostimulatory siRNA-driven innate immune activation by 2'-modified RNAs. **Biochem Biophys Res Commun** 361, 122-6.

Shakhar Guy B-ES (**2003**). Potential Prophylactic Measures Against Postoperative Immunosuppression: Could They Reduce Recurrence Rates in Oncological Patients? . **Annals of Surgical Oncology** 10, 972-92.

Sondak VK, Liu PY, Tuthill RJ, et al (**2002**). Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumour vaccine: Overall results of a randomized trial of the Southwest Oncology Group. **J Clin Oncol** 20, 2058–66,

Sobol RE, Fakhrai H, Shawler D, et al (**1995**). Interleukin-2 gene therapy in a patient with glioblastoma. **Gene Ther** 2, 164–7.

Shibata T GAJ, Brown J M (**2000**). Development of a hypoxia-responsive vector for tumor-specific gene therapy. **Gene therapy** 7, 493-8.

Tatum JL, Kelloff GJ, Gillies RJ, et al (**2006**). Hypoxia: importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. **Int J Radiat Biol** 82, 699-757.

Tlsty TD, Hein PW (**2001**). Know thy neighbor: stromal cells can contribute oncogenic signals. **Curr Opin Genet Dev** 11**,** 54-9.

Trédan O GC, Patel K, Tannock IF (**2007**). Drug resistance and the solid tumor microenvironment. **J Natl Cancer Inst** 99, 1441-54.

Tamada K, Tamura H, Flies D, et al (**2002**). Blockade of LIGHT/LTbeta and CD40 signaling induces allospecific T cell anergy, preventing graft-versus-host disease. **J Clin Invest** 109**,**549-57.

Van Pel A, Boon T (**1982**)**.** Protection against a nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis. **Proc Natl Acad Sci USA** 79**,** 4718-22

von Euler H, Sadeghi A, Carlsson B, et al (**2008**). Efficient adenovector CD40 ligand immunotherapy of canine malignant melanoma. **J Immunother** 31, 377-84.

Wu W, Luo Y, Sun C, et al (**2006**). Targeting cell-impermeable prodrug activation to tumor microenvironment eradicates multiple drug-resistant neoplasms. **Cancer Res** 66:970-80.

Welters MJ, Kenter GG, Piersma SJ, et al (**2008**). Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. **Clin Cancer Res** 14, 178-87.

Wagner E (**2007**). Converging Paths of Viral and Non-viral Vector Engineering. **Molecular Therapy** 16, 1-2.

Wolff JA, Malone RW, Williams P, et al (**1990**). Direct gene transfer into mouse muscle in vivo. **Science** 247, 1465–8,.

Wolfers J, Lozier A, Raposo G, et al (**2001**). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. **Nat Med** 7, 297–303.

Yang W, Chen PW, Li H, Alizadeh H, Niederkorn JY (**2008**). PD-L1: PD-1 Interaction Contributes to the Functional Suppression of T-Cell Responses to Human Uveal Melanoma Cells In Vitro. **Invest Ophthalmol Vis Sci** 49**,** 2518-25.

Zitvogel L, Regnault A, Lozier A, et al (**1998**). Eradication of established murine tumors using a novel cell-free vaccine: Dendritic cell-derived exosomes. **Nat Med** 4, 594–600.