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# On CD4$^+$ T Lymphocytes in Solid Tumours

PER MARITS



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2007

ISSN 1651-6206
ISBN 978-91-554-7034-0
urn:nbn:se:uu:diva-8325

Dissertation presented at Uppsala University to be publicly examined in Sal X, Universitetshuset, Uppsala, Friday, December 14, 2007 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

**Abstract**
Marits, P. 2007. On $CD4^+$ T Lymphocytes in Solid Tumours. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 295. 68 pp. Uppsala. ISBN 978-91-554-7034-0.

This thesis deals with recognition and elimination of tumours by T lymphocytes and their use in adoptive immunotherapy.

The first tumour-draining lymph node; the sentinel node, is identified by peritumoural injection of a tracer. This is the hypothesised location for the activation of tumour-reactive lymphocytes. Accordingly, proliferation and IFN-γ production in response to autologous tumour extract was detected in sentinel nodes from patients with colon and urinary bladder cancer. Reactivity in metastatic nodes was generally lower or absent, but the non-responsiveness could be subdued in long-term cultures by addition of tumour antigen and IL-2. A novel padlock-probe based method was developed for measuring the T cell receptor Vβ repertoire. Common Vβ gene expansions were detected in tumour-infiltrating lymphocytes and sentinel nodes. Thus, tumour antigens are recognised in sentinel nodes by Th1 lymphocytes, resulting in a clonally expanded cell population that can be further propagated *ex vivo*.

Regulatory T cells (Tregs) may contribute to tumour-induced immunosuppression. Immunohistochemical stainings against the pan-T cell marker CD3 and Treg marker FOXP3 was performed on tumour tissue from 20 historical urinary bladder cancer patients. The ratio of $FOXP3^+$ to $CD3^+$ cells was lower in patients alive 7 years post-cystectomy, suggesting that Tregs in bladder cancer have prognostic implications.

Lymphocytes were isolated from sentinel nodes from sixteen patients with advanced or high-risk colon cancer. *In vitro* expansion with addition of autologous tumour extract and IL-2 mainly promoted the outgrowth of $CD4^+$ Th1 lymphocytes, which were safely re-transfused to the patients. Four patients responded with complete tumour regression. Survival time in the Dukes' D patients was significantly increased compared with conventionally treated controls (2.6 versus 0.8 years; p=0.048).

In conclusion, human solid tumours are recognised in sentinel nodes and *in vitro* expanded sentinel node-acquired $CD4^+$ T lymphocytes seem useful in the treatment of patients with disseminated cancer.

*Keywords:* Tumour immunology, T lymphocytes, sentinel node detection, colon cancer, urinary bladder cancer, adoptive immunotherapy

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ISSN 1651-6206
ISBN 978-91-554-7034-0
urn:nbn:se:uu:diva-8325 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8325)

**To my Mother**

# List of papers

This thesis is based on the following papers, which will be referred by their Roman numerals:

I Marits, P., Karlsson, M., Dahl, K., Larsson, P., Wanders, A., Thörn, M., and Winqvist, O. (2006). Sentinel node lymphocytes: tumour reactive lymphocytes identified intraoperatively for the use in immunotherapy of colon cancer. Br. J. Cancer. 94, 1478-1484.

II Marits, P., Karlsson, M., Sherif, A., Garske, U., Thörn, M., and Winqvist, O. (2006). Detection of immune responses against urinary bladder cancer in sentinel lymph nodes. Eur. Urol. 49, 59-70.

III Baner, J., Marits, P., Nilsson, M., Winqvist, O., and Landegren, U. (2005). Analysis of T-cell receptor V beta gene repertoires after immune stimulation and in malignancy by use of padlock probes and microarrays. Clin. Chem. 51, 768-775.

IV Marits, P., Winerdal, M., Tolf, A., Thörn, M., Sherif, A., and Winqvist, O.: Tumour infiltrating $FOXP3^{+}$ regulatory T cells in urinary bladder cancer. Manuscript.

V Karlsson, M., Marits, P., Dahl, K., Dagöö, T., Enerbäck, S., Thörn, M., and Winqvist, O. Sentinel node $CD4^{+}$ Th1-cells induce tumour regression in humans. Submitted manuscript.

# Contents

# Abbreviations

| | |
|---|---|
| APC | Antigen presenting cell |
| BCG | Bacillus Calmette-Guérin |
| BSA | Bovine serum albumin |
| CCL | CC-chemokine ligand |
| CCR | CC-chemokine receptor |
| CD | Cluster of differentiation |
| CEA | Carcino embryonic antigen |
| DC | Dendritic cell |
| cDNA | Complementary deoxyribonucleic acid |
| EDTA | Ethylenediamine tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FOXP3 | Forkhead/winged-helix protein |
| Gp100 | Glycoprotein 100 kilodalton |
| HEV | High endothelial venule |
| HLA | Human leukocyte antigen |
| IFN | Interferon |
| IL | Interleukin |
| LAK | Lymphokine-activated killer |
| MAGE-1 | Melanoma antigen-1 |
| MART-1 | Melanoma antigen recognised by T cells-1 |
| MHC | Major histocompatibility complex |
| NK | Natural killer |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| RAG | Recombination-activating gene |
| mRNA | Messenger ribonucleic acid |
| STAT | Signal transducer and activator of transcription |
| T-bet | T-cell-specific T-box transcription factor |
| TCR | T cell receptor |
| TGF-β | Tumour growth factor β |
| Th | T helper |
| TIL | Tumour-infiltrating lymphocyte |
| TNF-α | Tumour necrosis factor-α |
| Treg | Regulatory T cell |
| VDJ | Variable – diversity – joining |

# Introduction

The main function of the immune system is to protect the the human body against potentially harmful microorganisms, and it can be divided into two parts; the innate and the adaptive arm. Innate immunity consists of anti-microbial proteins and phagocytic cells, which together provide a first line of defence against invading pathogens. Innate immune cells recognise conserved pathogen-associated molecules by means of germ-line encoded receptors. Adaptive immunity, mediated by lymphocytes, is characterised by the ability to respond to virtually any foreign molecule and also by displaying immunological memory, i.e. mounting a more rapid and efficient response upon re-encounter with a pathogen.

Each lymphocyte is equipped with a unique cell surface receptor, as originally proposed by Burnet. When the receptor binds a foreign antigen, the cell is activated and divides to form a clone of identical cells, capable of eradicating the pathogen. Following the primary response, some of the antigen-specific cells persist as long-lived memory cells which respond more rapidly and efficiently when re-triggered by the antigen. This concept, referred to as the clonal selection theory has formed a basis for immunological thinking during the second half of the $20^{th}$ century. Another striking feature of adaptive immunity is self – non-self discrimination; i.e. the lymphocytes remain non-responsive to the proteins and tissues of the host despite the enormous diversity of their antigen receptors. This is achieved by removal of autoreactive cells from the repertoire during development. However, a more elusive question remains: Are the mature lymphocytes able to recognise transformed cells of the body, i.e. cancer cells, which are neither foreign, nor truly self?

This latter aspect of self – non-self discrimination, termed tumour immune surveillance, is the general theme of this thesis. The focus of my work has been one of the principal cells of the adaptive immune system, namely the $CD4^+$ T lymphocyte and its role in human solid tumours of the colon and urinary bladder.

In the following pages I will give an overview of T lymphocyte development and function, followed by an introduction to the concept of tumour immune surveillance and immunotherapy of cancer.

# T cell maturation and function

T and B lymphocytes, as well as Natural Killer (NK) cells, originate from a common lymphoid progenitor cell in the bone marrow. NK cells are considered innate effector cells, whereas T and B cells together constitute the adaptive immune system. The B lymphocytes provide humoral immunity by recognition of soluble antigen and secretion of antibodies. T cells, instead, recognise antigens as peptides bound to the polymorphic cell-surface molecules encoded by the major histocompatibilty complex (MHC). Two principal T cell subsets can be distinguished; $CD8^+$ cytotoxic T cells and $CD4^+$ T cells. The latter are commonly termed helper T cells due to their role in augmenting antibody production by B cells and cytotoxic T cell responses. However, as addressed below, the CD4 subset also comprises regulatory T cells with immunosuppressive properties. The T cell precursors leave the bone marrow and complete their maturation in the thymus, during which they start to express their antigen receptor.

## The T cell receptor

There are approximately $10^{12}$ T lymphocytes in the human body and estimates of the number of distinct T cell specificities range between $2.5x10^7$ – $1.1x10^8$ (Arstila et al., 1999; Ogle et al., 2003). Evidently, this enormous diversity cannot be achieved by germ-line encoded receptor genes. Instead, the immune system utilise gene fragments which are combined during lymphocyte maturation to encode functional receptor genes (Figure 1).

The T cell receptor (TCR) for antigen recognition is composed of two polypeptide chains. The majority of mature T cells express a TCR composed of an α- and a β- chain, non-covalently associated with the CD3 protein complex, which mediate signal transduction upon antigen binding. The TCR genes are composed of V and J segments for the α-chain and V, D, and J segments for the β-chain. For the human α-chain, there are 43-45 functionally expressed V genes and 50 functional J genes, whereas the β-chain locus comprises at least 43-45 functional V genes which can be combined with 2 D and 13 J genes (IMGT, the international ImMunoGeneTics information system http://imgt.cines.fr, accessed September 7th 2007). The receptor diversity is further increased by imprecise joining of the gene segments and insertion of non-templated nucleotides at the V(D)J junctions; and finally by the combination of an α- and a β-chain in each T cell (Davis and Bjorkman, 1988), resulting in a number of possible variants that exceeds the number of T cells in the body by several orders of magnitude. The recombination process occurs during the thymic maturation in a highly ordered fashion. The β-chain locus is rearranged first and the appearance of its protein product on the cell surface, in combination with a surrogate pre-Tα-chain, is required for the thymocyte to continue with α-chain rearrangement. Concomitantly,

the CD4 and CD8 molecules are both expressed on the cell surface. When a complete αβ TCR is expressed, the lymphocyte is positively selected for its ability to bind the MHC molecules of the host and commit to either the $CD4^+$ or $CD8^+$ lineage. In addition, the T cell is negatively selected, i.e. deleted, if it recognises self-peptide MHC complexes with too high affinity (Starr et al., 2003).

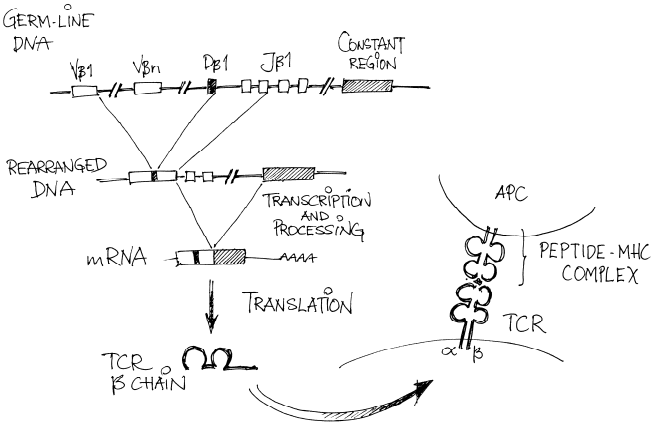


*Figure 1.* TCR gene rearrangement and MHC-restricted antigen recognition. In germ-line DNA, the TCR α- and β-chain loci contain multiple discrete gene segments, which are rearranged during T cell maturation to a functional gene for the variable domains of the TCR. For the β-chain (shown in the figure above), this entails combining one of each of V, D and J gene segments. The VDJ gene is transcribed to mRNA and spliced to a gene segment encoding the constant domain. Upon translation to protein, this generates the TCR β-chain, which pairs with an α-chain soon after synthesis. The TCR αβ-heterodimer appear at the cell surface and recognises antigenic peptides presented on MHC molecules. In the picture, an MHC class II molecule is shown. The CD4 co-receptor on the surface of the T-cell, which participates in MHC II-binding, is omitted for artistic reasons.

## T cell antigen recognition

The MHC locus, first identified in the context of transplantation in mice, encodes highly polymorphic proteins, referred to as Human Leukocyte Antigens (HLA) in man. The requirement for combined recognition of the MHC molecule and an antigenic peptide was discovered by Zinkernagel and Doherty (1974) and the crystal structure of the TCR bound to a peptide-MHC complex has been resolved (Garcia et al., 1996). Two types of MHC proteins participate in antigen presentation to T cells, designated MHC class I and II. MHC class I molecules are composed of a membrane-bound α-chain associ-

ated with the β2 microglobulin protein whereas class II proteins consist of an α- and a β-chain. Both MHC molecules share the common structural feature of a cleft on top of the molecule, accommodating the antigenic peptide displayed to the T cell. MHC class I molecules are present on all nucleated cells and loaded with peptides derived from degraded intracellular proteins, which are presented to $CD8^+$ T cells. MHC class II molecules, by contrast, are mainly expressed by certain so-called professional antigen-presenting cells (APC), including macrophages, dendritic cells and B cells. These cells endocytose extracellular material and digest it to peptides, which are directed to the class II pocket and displayed on the cell surface for recognition by $CD4^+$ T cells. In addition, certain APC are capable of presenting fragments from extracellular sources on their MHC class I molecules, a process known as cross-presentation (Heath and Carbone, 2001).

It has been demonstrated that a $CD4^+$ T cell can detect even a single specific peptide bound to an MHC molecule, as measured by signal transduction events inside the cell, and maximal activation occurs at 8-10 peptide-MHC (pMHC) complexes (Irvine et al, 2002). Furthermore, efficient target cell killing by cytotoxic $CD8^+$ T cells was achieved at as few as three pMHC complexes (Purbhoo et al., 2004).

## Lymphatic recirculation

Mature T cells exit the thymus and begin a continuous recirculation from the blood through peripheral lymphoid tissues and back (Gowans and Knight, 1964).

The lymphatic system, first described by Olof Rudbeck in the mid $17^{th}$ century, consists of lymphatic vessels which collect extracellular fluid from the tissues. The vessels interconnect, becoming progressively larger until they coalesce into the thoracic duct, which empties its content into the left subclavian vein. By cannulating the thoracic duct in mice, investigators observed that antigen-specific cells transiently disappeared from the lymph, suggesting that they were selectively recruited into secondary lymphoid organs (Sprent et al., 1971). In these studies, the antigen was administered intravenously and the site for sequestration was most likely the spleen. By contrast, lymph nodes, distributed along the lymphatics, receive antigens from peripheral tissues and are therefore of special interest with regards to immune surveillance of solid tumours.

Recirculating lymphocytes enter the lymph node from the blood. Lymphocyte transmigration through the wall of the high endothelial venules (HEV) in lymph nodes is a highly regulated process, consisting of at least three distinct molecular events. First, tethering of the lymphocyte to the endothelium is mediated by binding of the lymphocytic cell-surface molecule CD62L (L-selectin) to carbohydrate ligands present on HEV. Pushed forward by the blood stream, the cell begins rolling along the vessel wall. Sec-

ond, the lymphocyte arrests upon encounter of a chemotactic signal, which activates integrin-mediated adhesion and, finally, transmigration (Springer, 1994). Migration of the lymphocytes within and their exit from the lymph node is also regulated through cell surface receptors and chemotactic signals. Accordingly, newly arrived T cells are guided to the T cell zones of the node mainly by the chemokine CCL 21, through binding of the receptor CCR7 on the lymphocytes (Förster et al., 1999), whereas T cell egress from the node is dependent on binding of sphingosine-1-phosphate to its cell-surface receptor. This receptor is transiently downregulated upon T cell activation and thereby provides a mechanism for retention of antigen-specific cells in the node (Matloubian et al., 2004).

## Activation of T cells in the lymph node

Within the specialised microenvironment of the lymph node, lymphocytes encounter tissue-derived antigens, both foreign and self molecules, displayed by professional antigen-presenting cells (APC). The exact phenotype of the APC responsible for $CD4^+$ T cell activation is not completely defined. Some early experimental findings suggested a role for B cells in this process (Ron and Sprent, 1987) but in light of more recent data, a strong case can be made for the dendritic cell (DC) as the main APC in the T cell areas of the lymph node.

In the resting state, DCs are efficient phagocytes but upon activation, which occur in response to inflammatory or microbial stimuli, they mature to potent antigen-presenting cells. Thus, phagocytosis is shut down and the expression of MHC and co-stimulatory molecules, for example B7-1(CD81) and B7-2(CD86), increases. The mature DC then migrates to the T cell areas of the tissue-draining lymph node to activate T cells (Jenkins et al., 2001). *In vivo* imaging of a T cell response under inflammatory conditions has shown that an antigen-specific naïve $CD4^+$ T cell first makes multiple, brief contacts with neighbouring antigen-presenting DCs, showing the first signs of activation such as cell-surface expression of CD69. Then, it forms a more long-lasting (>1 hour) conjugate with a DC and, following dissociation, the T cell starts to divide, which indicates the beginning of the clonal expansion phase (Miller et al., 2004). The outcome of DC – T cell interaction is greatly influenced by co-stimulatory signals, especially those transmitted through the CD28 receptor by the B7 molecules. In their absence, the activated T cells are rendered non-responsive to further antigen-stimulation, a phenomenon known as anergy (Ragazzo et al., 2001). By contrast, optimally activated $CD4^+$ T cells proliferate and differentiate into functional effector cells.

### *Effector and memory cells*

On the basis of cytokine secretion, murine T helper cells may be classified as type 1 (Th1) cells, secreting mainly IFN-γ, IL-12 and TNF-α, or type 2 (Th2)

cells, secreting IL-4, IL-5, and IL-13. (Mosmann et al., 1986). Albeit human T helper cells show certain heterogeneity with respect to their cytokine secretion profiles, the Th1/Th2 paradigm has been useful for revealing fundamental aspects of T helper cell functions. Th2 cells sustain humoral immunity, whereas Th1 cells promote cellular immunity by activating macrophages and providing co-stimulation to $CD8^+$ cytotoxic T cells. Accordingly, the type 1 response is considered the main effector mechanism in tumour immunity. The Th1/Th2 phenotype is established by signalling through cytokine receptors on the T cell, which initiates epigenetic changes involving covalent modifications of DNA and histones. In developing Th1 cells, the transcription factor T-bet is induced and mediates both transcription and remodelling of the IFN-γ gene. The cytokine profile is thereby conferred to the daughter cells during cell division in the clonal expansion phase (Murphy and Reiner, 2002).

Following antigen clearance the majority of effector cells undergo apoptosis, but some descendants from the effector cells survive as long-lived memory cells. Two major subtypes of memory T cells can be distinguished: central and effector memory cells. The former maintain expression of the chemokine receptor CCR7 and primarily reside in secondary lymphoid organs. They have limited immediate effector functions, but readily proliferate and differentiate into effector cells upon stimulation. The effector memory cells, by contrast, rapidly execute effector functions upon antigen stimulation, but have a limited capacity to divide. They lack CCR7 and are heterogeneous with respect to CD62L expression. Instead, they are equipped with receptors for access to inflamed tissues (Sallusto et al., 2004).

*$CD4^+$ T cell help to $CD8^+$ T cells*

Although cytotoxic T cells can be raised against certain bacteria and viruses in the absence of $CD4^+$ cells (Rahemtulla et al., 1991), several investigators have found that CD4 T cell help is necessary for strong $CD8^+$ T cell responses (Bennett et al., 1997; Ossendorp et al., 1998). It has been suggested that lack of $CD4^+$ T cell help may be compensated by strong signals from innate immune receptors. However, even the $CD8^+$ effector T cells raised under such conditions are unable to generate functional memory cells (Janssen et al., 2003). Thus, the $CD4^+$ T cells both augment the primary response as well as program the differentiation of the responding cytotoxic T cells into long-lived protective memory. The $CD4^+$ T cell provides this help, at least partially, through a dendritic cell that presents antigen to both the $CD4^+$ and $CD8^+$ T cell (Bennett et al., 1997) and, like most cellular interactions within the lymph node, this ménage à trois is assembled through chemokine signals attracting $CD8^+$ T cells to antigen-specific DC-$CD4^+$ T cell conjugates (Castellino et al., 2006). Altogether, much less is known of the cellular interactions occurring after the primary response, and the re-

quirements for $CD8^+$ effector and memory T cell generation in humans may be quite different from those observed in experimental mouse models.

## Regulatory T cells

Thymocytes that are activated by recognition of self are deleted during negative selection, but this process is imperfect and many autoreactive T cells are released into the circulation. Consequently, the immune system must employ additional mechanisms to maintain self – non-self discrimination, one being regulatory T cells (Tregs). A large number of T cell subpopulations with immunosuppressive properties have been described and a complete account for all of these is far beyond the scope of this thesis, but restricting the text to $CD4^+$ Tregs, mainly those of the naturally occurring, $FOXP3^+$ subset.
The presence of regulatory $CD4^+$ T cells was reported already in the 1960's, but a paucity of available surface markers precluded a more precise characterisation. In 1995, Sakaguchi and colleagues described a $CD4^+$ T cell subset in mice which expressed CD25; the α-chain of the IL-2 receptor, comprising 5-10% of the $CD4^+$ T cells in blood. When the total $CD4^+$ splenocytes from a healthy immunocompetent laboratory mouse was depleted of $CD25^+$ cells and then transferred into a T-cell deficient mouse, severe autoimmune destruction of several internal organs ensued, which could be prevented by co-transfer of small numbers of the $CD25^+$ $CD4^+$ T cells. It was further demonstrated that the cells developed in the thymus and suppressed the activation of effector T cells in a contact-dependent manner. Furthermore, they require activation through their TCR to be suppressive but then exert their suppressive functions antigen-independently. Later, the transcription-factor Foxp3 was identified as necessary and sufficient for the development and function of this Treg subset in mice (Sakaguchi, 2004).

Naturally occurring Tregs with similar properties also exist in humans and their importance is illustrated by the IPEX (immune dysfunction /polyendocrinopathy /enteropathy/ X-linked) syndrome which is caused by inactivating mutations in the *FOXP3* gene (Bennett et al., 2001). These patients develop intestinal inflammation, wide-spread autoimmunity and allergic manifestations, and often die in the first two years of life. Human naturally occurring Tregs have been described as the $CD25^{high}CD4^+$ cell population in peripheral blood (Baecher-Allan et al., 2001). However, CD25 is upregulated upon T cell activation, which precludes unambiguous identification of these Tregs on the basis of surface phenotype in humans, especially within lymph nodes and inflammatory environments. FOXP3 might represent a more reliable marker, but may also be induced upon activation of human – but not murine – non-regulatory T cells (Ziegler, 2006). There are conflicting data as to whether these induced $FOXP3^+$ T cells possess regulatory activity. In most studies, however, FOXP3 expression appeared transiently and a stable Treg phenotype was not obtained, as indicated by differ-

ential methylation of the *FOXP3* locus, in activated T cells with an induced FOXP3 expression, as compared with that of the naturally occurring Tregs (Baron et al., 2007; Jansson et al, submitted).

It has been suggested that thymocytes with a high affinity to self-peptide MHC complexes, but not high enough to be deleted, become Tregs (Jordan et al., 2001). Thymic expression of several tumour antigens has been detected (Kyewski and Klein, 2006), and Tregs specific for these antigens may be an obstacle to tumour immunotherapy. The role of $FOXP3^+$ Tregs in cancer will be further discussed in the following sections.

In addition to the thymus-derived Tregs, $CD4^+$ T cells may become suppressive upon antigen-stimulation in the periphery. Among the most well-characterised induced Treg subsets are Tr1 and Th3 cells. The former result from activation in the presence of IL-10, or upon stimulation by an IL-10 conditioned DC. They are antigen-specific and suppress both naïve and memory cell T responses by secretion of the immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β). Th3 cells have been induced in studies of oral tolerance, secrete mainly TGF-β and share many properties with the Tr1 cells (Roncarolo et al., 2001).

## Tumour immunology

The transformation of a normal cell into a malignant cancer cell is a multistep process, during which sequential genetic changes cause defects in the regulatory circuits which keep the cell in its physiological context. Six distinctive traits have been described as "the hallmarks of cancer": Self-sufficiency in growth signals, insensitivity to antigrowth signals, apoptosis resistance, limitless replicative potential, sustained angiogenesis and, finally, tissue invasion and metastasis. To enable all these requirements to be fulfilled within the life-time of an organism a seventh feature; genomic instability is needed. (Hanahan and Weinberg, 2000). As a consequence, a large number of mutated proteins arise during tumourigenesis, generating antigens which are "new" to the immune system and the cancer cell may thus be perceived as non-self.

The recognition and elimination of tumours by the immune system was discussed already in the beginning of the 20$^{th}$ century, but the idea was put into the context of the clonal selection theory by Lewis Thomas and later by Frank Macfarlane Burnet several decades later (Burnet, 1970). In its original version, tumour immune surveillance is postulated to be the main protective mechanism against cancer in long-lived animals. However, one of the obvious interpretations of the model, an increased cancer incidence in states of immunodeficiency was not supported by investigations of the immunodeficient *nude* mouse (Rygaard and Povlsen, 1974; Stutman, 1974); the significance and even existence of tumour immune surveillance was questioned.

During the last decade, genetically engineered mouse models have provided evidence in favour of the concept. By specifically deleting the genetic elements required for V(D)J recombination of the antigen receptor genes, mice with complete lack of all B and T cells are generated (RAG deficient mice), in contrast with the *nude* mouse strain, which do have some residual T and B cells. With increasing age, these gene-targeted mice develop spontaneous colon adenomas and adenocarcinomas of the intestine and lungs. Furthermore, when the RAG deficient mice were unable to mediate IFN-γ signalling due to a deletion of the signal transduction molecule STAT-1, additional tumours of the colon and breast develop (Shankaran et al., 2001). Importantly, these mice were raised under sterile conditions and infections or chronic inflammation as explanations for the increased tumour incidence were ruled out. A number of other genes encoding immune response-related molecules, including the cytokine IFN-γ and proteins involved in antigen-presentation, and lymphocyte cytotoxoicity, have been targeted in other studies (Dunn et al., 2004). Similarly, these mouse strains also display increased spontaneous tumour incidence, as well as increased sensitivity to chemical carcinogens. Notably, a large fraction of the spontaneous malignancies were lymphomas, suggesting that tumour immune surveillance may be at its best within the haematopoietic system. It has even been proposed that tumours that grow strictly outside secondary lymphoid organs are ignored by the immune system (Ochsenbein et al., 2001). However, this is contradicted by the findings of increased incidences of solid tumours in the lungs and intestine of RAG deficient mice. In addition, carcinogen-induced sarcomas from these mice were rejected when transplanted into normal, but not into RAG deficient mice, whereas sarcomas from immunocompetent mice grew progressively in all the recipients (Shankaran et al., 2001), indicating that the cancer cells had been shaped by the immune system to adopt a less immunogenic phenotype.

## Evidence for tumour immune surveillance in humans

Hereditary immunodeficiency in humans give rise to an increased risk for malignancies, but primarily due to virally induced carcinogenesis. As inefficient elimination of the virus is a possible explanation, this does not give unequivocal support to the immune surveillance hypothesis in its original sense. By contrast, renal transplant patients, receiving immunosuppressive drugs, have an increased incidence of cancers, including solid tumours with no established association to viral infection (Vajdic et al., 2006).

Prominent infiltration of lymphocytes are frequently seen in solid tumours. The extent of T cell-infiltration has been correlated with a favourable prognosis in several human cancers, including colon (Pagès et al., 2005) and urinary bladder cancer (Lipponen et al., 1993). Furthermore, in the study by Pagès et al., a clear benefit of a Th1 response was demonstrated by analysis

of IFN-γ and T-bet expression in tumour biopsies. A third argument for the presence of immunosurveillance in humans is spontaneous tumour regression, which has been estimated to occur in between 1 out of 60,000 and 100,000 people with cancer. The most common malignancies in this context are malignant melanoma, renal cell cancer, low-grade non-Hodgkins's lymphoma, chronic lymphocytic leukaemia and neuroblastoma in children, which together comprise about two thirds of cases (Kappauf et al., 1997). In malignant melanoma, an immunological background of a regression has been formally demonstrated (Mackensen et al., 1994).

The molecular basis for immune cell recognition of tumours has been elucidated during the last decade, through the discovery of tumour antigens and isolation of tumour-associated lymphocytes with specific reactivity against autologous cancer cells (addressed below). These findings provide definitive support for an operating immune surveillance in cancer patients and form the basis for tumour immunotherapy.

## How to reject a tumour

What mechanism maintains immune surveillance? Given the results obtained in the RAG and STAT-1 deficient mice, adaptive immunity and the cytokine IFN-γ are apparently important, implicating a Th1 response. Supporting this conclusion are data from cancer patients (Pagès et al., 2005), and results obtained in STAT-6 deficient mice. These animals, which preferentially polarise their $CD4^+$ T cells into a Th1, rather than a Th2 response, rejected poorly immunogenic mastocytoma which grew progressively in wild-type mice (Kacha et al., 2000). Innate immune cells are likely to contribute to immune surveillance, as indicated by the increased tumour susceptibility in NK cell deficient mice (Smyth et al., 2000). NK cells are capable both of IFN-γ secretion and tumour cell killing through cytolysis. In addition, the cytotoxic functions of macrophages are activated by IFN-γ. Myeloid cells are present within many solid tumours, but their role is complex. In fact, most data suggest that the main functions of tumour-infiltrating macrophages are immunosuppressive (Sica and Bronte, 2007).

Of more significance for immune surveillance are the actions of IFN-γ on lymphocytes and tumour cells. The cytokine diverts activated $CD4^+$ T helper cells to the Th1 lineage and promotes the development of cytotoxic $CD8^+$ T cells. Effects on tumour cells include inhibition of proliferation and tumour-induced angiogenesis, as well as up-regulation of proteins involved in antigen processing and presentation, including MHC class I molecules (Ikeda et al., 2002). IFN-γ can also induce aberrant MHC class II expression, thereby making tumour cells visible to $CD4^+$ T cells. Interestingly, MHC class II expression has been identified as a positive prognostic marker in colon cancer (Lovig et al., 2002).

## Tumour antigens

An MHC class I peptide derived from the protein MAGE-1 and recognised by a $CD8^+$ T cell clone from a melanoma patient which displayed cytotoxic activity with the autologous tumour cells was the first tumour-antigen derived T cell epitope to be identified (van der Bruggen et al., 1991). The first MHC class II-restricted antigen was tyrosinase, also identified in a T cell line from a patient with malignant melanoma (Topalian et al., 1994).

Tumour antigens are usually grouped into four different categories:

1. Cancer-testis antigens are, as the name implies, expressed in the testis and then reexpressed in tumours of different origin. MAGE-1, belongs to this large family of proteins. Tumours can be divided into high, intermediate, or low expressing. Melanoma and urinary bladder cancer are high expressing tumours as more than half of the known cancer-testis antigens are expressed in 20-70% of tumours, whereas colon cancers belong to the low expressing group (Scanlan et al., 2004).
2. Differentiation antigens: Proteins shared between tumours and the normal tissue from which the tumour has developed. Tyrosinase is a typical differentiation antigen and the melanocyte has provided tumour immunology with a large number of antigens belonging to this category. Another example is carcinoembryonic antigen (CEA), used as a tumour marker in colon cancer, expressed both in normal epithelia and the majority of gastrointestinal adenocarcinomas.
3. Widely occurring, overexpressed tumour-associated antigens: These proteins are detected in a wide variety of tumours of different origin and may be present in many normal tissues, but at lower levels.
4. Unique and shared tumour-specific antigens: antigens that have arisen due to point-mutations in tissue proteins. Some of these antigens are associated with the malignant transformation and have implications for tumour growth and/or patient survival.

To date, a large number of tumour antigens have been described in tumours of different origin, although with a strong preponderance for malignant melanoma and for antigens recognised by $CD8^+$ T cells.

The most successful method to identify MHC class I-restricted antigens has been by screening of cDNA libraries derived from the autologous tumour cells with tumour-reactive lymphocytes (van der Bruggen et al., 1991). Antigen-presentation on MHC class II molecules involves endocytosis, processing and loading in specialised intracellular compartments, unique to APC. The expression screening approach was therefore not directly applicable for $CD4^+$ T cell antigens, which delayed their discovery. An elegant solution was presented by Wang and colleagues (2003), who managed to direct the expressed tumour-derived proteins to the lysosomal pathway in MHC-

matched APC, followed by stimulation and screening of $CD4^+$ T cell lines for specific cytokine secretion. This method, in conjunction with other novel techniques, has spurred the identification of class II-restricted peptides.

Defined tumour antigens harbouring MHC class II restricted epitopes are listed in table I. The table was compiled from: http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm, updated on March 12, 2007. The epitopes that are included in the list have fulfilled the following requirements: (a) isolation of stable human T lymphocyte clones or lines recognising the peptide, (b) identification of the peptide recognised by the T cells, (c) identification of the HLA presenting molecule, (d) there is evidence that the peptide is processed and presented by tumour cells or rather, in the case of $CD4^+$ T cell epitopes, by APC, (e) a certain level of tumour- or tissue-specificity have been documented. Using less stringent criteria, additional epitopes may be included but the overall picture will remain unchanged: excluding malignant melanoma; the number of MHC class II-restricted antigens available for specific immunotherapy of solid tumours is very limited.

Table 1. *MHC class II-restricted tumour antigens in solid tumours*

| Antigen | No of epitopes | Tumours |
|---|---|---|
| **Cancer-testis antigens** | | |
| NY-ESO-1 | 12 | Melanoma, myeloma, lung cancer, esophageal SCC, head-and-neck SCC, bladder, prostate and breast carcinomas |
| LAGE-1/CAMEL | 6 | Same as NY-ESO-1 |
| MAGE-A1 | 1 | Melanoma, myeloma, lung cancer, esophageal and head-and-neck SCC, bladder cancer |
| MAGE-A2 | 2 | Same as MAGE-A1 |
| MAGE-A3 | 9 | Same as MAGE-A1 |
| MAGE-A6 | 1 | Same as MAGE-A1 |
| MAGE-A12 | 1 | Same as MAGE-A1 |
| SSX-2 | 4 | Melanoma, head-and-nek SCC, ovarian, colon and breast cancer, sarcomas, some leukemias and lymphomas |
| SSX-4 | 7 | Same as SSX-2 |
| TRAG-3 | 3 | Melanoma, breast, lung, esophagus, liver, biliary, gastric, colon and ovarian cancers |
| **Differentiation antigens** | | |
| CEA | 11 | Colon carcinoma, other adenocarcinomas |
| Gp100 | 5 | Melanoma |
| MART-1/Melan-A | 6 | Melanoma |
| Tyrosinase | 3 | Melanoma |
| TRP1/p75 | 2 | Melanoma |
| Kallikrein A | 3 | Prostate |

| Antigen | No of epitopes | Tumours |
|---|---|---|
| **Widely expressed antigens** | | |
| Cyclin D1 | 1 | |
| EphA3 | 1 | Melanoma, sarcomas, lung cancer and renal cell carcinoma |
| MUC1 | 1 | Breast and ovarian cancer, myeloma, B-cell lymphoma |
| p53 | 2 | Several |
| Telomerase | 2 | Several |
| WT1 | 1 | Gastric, colon, lung, breast, ovary, uterine, thyroid, and hepatocellular cancer |
| **Tumour-specific antigens** | | |
| ARTC-1 | 1 | Melanoma |
| B-raf | 1 | Melanoma |
| CDC27 | 1 | Melanoma |
| Fibronectin | 1 | Melanoma |
| Neo-PAP | 1 | Melanoma |
| PTPRK | 1 | Melanoma |
| TP1 | 1 | Melanoma |
| LDLR/FUT | 2 | Melanoma |
| COA-1 | 2 | Colon cancer |
| TGFβRII | 1 | Colon cancer |

Abbreviations in Table 1: NY-ESO-1=New York esophageus-1, LAGE=L antigen, MAGE=Melanoma antigen, CAMEL=CTL-recognised antigen on melanoma, SSX=sarcoma, synovial, X-chromosome-related, TRAG-3=Taxol-resistant-associated protein-3, CEA=Carcinoembryonic antigen, Gp100=Glycoprotein 100 kDa, MART-1/Melan-A=Melanoma antigen recognised by T cells-1/melanoma antigen A, TRP=Tyrosinase-related peptide, EphA3=Ephrin type-A receptor 3, HER-2/neu=Human epidermal receptor 2/neurological, MUC1=Mucin 1, WT1=Wilms' tumour gene 1, ARTC-1=Antigen recognized by Treg cells-1, CDC27=Cell division cycle 27, Neo-PAP=Neo-poly(A) polymerase, PTPRK=Receptor-type protein-tyrosine phosphatase kappa, TP1=Triosephosphate isomerase, LDLR/FUT1=Low density lipid receptor/GDP-l-fucose:β-d-galactosidase 2-α-fucosyltransferase, COA-1=colorectal tumor-associated antigen-1, TGFβRII=Transforming growth factor β receptor 2, SCC=Squamous cell carcinoma

## Immune escape

As discussed above, an intact immune system appears to shape malignant cells to become less immunogenic. The theoretical background can be conceived as the combination of a selection pressure imposed on the tumour by continuous immune surveillance and the inherent genetic instability of cancer cells (Hanahan and Weinberg, 2000). The ultimate consequence may be a tumour capable of unrestricted growth in an immunocompetent host – immune escape. A vast array of strategies employed by tumours to avoid detection and/or elimination have been described. Sometimes phenomena that are intrinsic to the immune system, such as ignorance and central tolerance, are included in immune escape. This overview, being far from complete, is mainly devoted to mechanisms initiated by the tumour.

*Altered antigen presentation*

Since MHC class I expression is a *sine qua non* for recognition by cytotoxic $CD8^+$ T cells, downregulation of these molecules may represent a straightforward strategy for tumours to avoid T-cell recognition and killing. Downregulated or lost expression of MHC class I molecules is common in human solid tumours; reported in 70-80% of colon cancers and 40-60% of urinary bladder cancers (Natali et al., 1989). An alternative strategy for tumours to escape recognition is downregulation of tumour antigens, resulting in the outgrowth of so-called antigen-loss variants. A demonstration of the relevance of both these theoretically appealing immune escape mechanisms was presented by Khong et al. (2004) in a melanoma patient, who first had responded to immunotherapy with antigen-specific clonal expansion of T cells and tumour regression. Later, upon disease progression, one metastatic lesion had lost the restricting MHC class I molecule, while another concurrent lesion had lost tumour antigen expression.

*Tumour cell resistance*

Immune surveillance rely, at least in part, on direct IFN-γ-mediated effects on the tumour cell, as demonstrated by the resistance of IFN-γ receptor deficient tumours to immune mediated rejection. Accordingly, several human adenocarcinoma cell lines were found to be insensitive to the cytokine due to downregulation of receptor signalling molecules downstream of the IFN-γ receptor (Ikeda et al., 2002).

Death receptor-mediated cellular cytotoxicity represents another threat to the integrity of the tumour cell, which can be circumvented by downregulating components of signal transduction of the apoptosis machinery (Igney and Krammer, 2002). Another resistance mechanism is tumour counterattack: acquired expression of the death receptor ligand FasL on tumour cells may induce apoptosis in activated T cells, which express the Fas receptor. Several human tumours have been reported to express FasL, and experimental evidence in favour of the theory has been obtained *in vitro*. However, the relevance of this phenomenon for immune escape *in vivo* has been questioned (Restifo, 2002).

*The immunosuppressive tumour microenvironment*

It has long been recognised that cancer patients show evidence of general immunosuppression, being a direct consequence of tumour-derived factors. For example, leukocytes in peripheral blood from colon cancer patients display reduced cytokine secretion upon stimulation which is normalised following surgical resection of the primary tumour (Heriot et al., 2000). Furthermore, the development of carcinogen-induced tumours in mice is associated with diminished responses of both T and NK cells, with reduced ability to secrete Th1 cytokines and downregulation of CD3ζ chain, a component of

the signal transduction pathway from the T cell receptor. Reduced levels of the CD3ζ chain has also been observed in lymphocytes infiltrating human colon cancers and the alterations become more pronounced during tumour progression (Kiessling, 1997). The emerging scenario is an immunosuppressive milieu created by the tumour which restrains immunosurveillance and the molecular basis for these alterations is multifactorial.

For example, high levels of IL-10 and TGF-β are often found in human malignancies (O'Hara et al., 1998; Shariat et al., 2001). Both cytokines inhibit T cell proliferation and effector functions. In addition, IL-10 impinge on APC, reducing their expression of MHC and co-stimulatory molecules. As discussed above, this may result in T cell anergy or induction of Tr1 cells (Roncarolo et al., 2001). Furthermore, tumours can express the enzyme indoleamine-2, 3-dioxygenase (IDO). The metabolic activities of this enzyme deplete tryptophan from the microenvironment, which in turn induces proliferative arrest in activated T cells (Uyttenhove et al., 2003).

Haematopoietic cells of the myeloid lineage, predominantly with a macrophage-like phenotype, are found within tumours of both mice and men. They have been actively recruited by tumour-derived chemotactic factors to secrete growth factors and promote angiogenesis. In addition, they have immunosuppressive properties, in part through secretion of IL-10 and TGF-β, and may impose a Th2 skewing on adaptive immune responses (Sica and Bronte, 2007).

*T cell-mediated immune escape?*

It was demonstrated almost three decades ago that a population of T cells from tumour-bearing mice were capable of suppressing immune-mediated rejection of an established tumour (Berendt and North, 1980). With the characterisation of the Treg population in the mid 90's, more elaborate experimental testing of this observation became possible. It was shown that effective tumour immunity in mice bearing progressively growing tumours could be evoked by depletion of $CD25^+CD4^+$ T cells by anti-CD25 antibody treatment (Sakaguchi, 2004). The relevance for human cancer was indicated by increased numbers of $FOXP3^+$ Tregs in patients with different solid tumours. A hallmark paper in this setting is the study of ovarian cancer patients by Curiel and colleagues (2004), who demonstrated an increased number of $FOXP3^+$ Tregs in the tumour and malignant ascites. This was a result of production of the chemokine CCL22 by tumour cells and tumour-infiltrating macrophages, which induced specific migration of Tregs to the tumour. In addition, the extent of Treg infiltration correlated with reduced survival and these findings have also been extended to other solid tumours (Bates et al., 2006; Kobayashi et al., 2007). To date, two tumour antigen-derived epitopes recognised by $FOXP3^+$ Tregs have been described; from the proteins LAGE-1 and ARTC-1 (Table 1), both in the context of malignant melanoma (Wang et al., 2004; Wang et al., 2005).

In addition to the $FOXP3^+$ Tregs, other regulatory subsets are likely to be involved in suppression of tumour-immunity. In the study by Berendt and North (1980), the suppressive function of the T cells developed gradually following tumour implantation, implicating de novo induced Tregs. With more recent data at hand, several candidate populations can be envisioned. Considering the abundant sources of IL-10 in the tumour microenvironment, Tr1 cells are strong suspects, while another explanation is infectious tolerance; a mechanism whereby the suppressive activity of Tregs is transferred to naïve $CD4^+$ T cells upon simultaneous TCR-dependent activation (Jonuleit et al., 2002). However, human Treg subsets show considerable overlap and their interrelationship is still obscure.

## Immunotherapy of cancer

The global impact of cancer cannot be exaggerated; approximately 25 million people are living with cancer today. Each year over 10 million new cases are to be expected and 7 million patients will die from malignant disease (Parkin et al., 2005).

Three major strategies are used to treat cancer: surgical resection of the tumour, exposure to ionising radiation and administration of cytotoxic drugs. For the vast majority of solid tumours, surgery is the primary treatment modality and may be sufficient in localised disease. In the setting of metastatic tumour growth the prognosis is poor – survival rates can be improved by the addition of radio- and/or chemotherapy but even in the developed world the likelihood of a cure remains less than 50% for many solid tumours. (Parkin et al., 2005). Clearly, there is room for improvement and hopes have been raised that immunotherpy can contribute to this; to become the fourth treatment modality of malignant disease.

Attempts to use the immune system to treat cancer were made long before the theory of tumour immune surveillance had been formulated. In 1891, the surgeon William B. Coley conducted the first systematic study in the field. Based upon observations of spontaneous tumour regressions in patients who developed bacterial infections, he inoculated streptococci in three patients with malignant tumours. All three displayed tumour regressions, but unfortunately two of the patients died of the infection. He refined his concept, including the use of heat-killed bacteria, to produce Coley's toxin, which was administered to large numbers of cancer patients and accompanied by occasional tumour regressions (McCarthy, 2006). A descendant of Coley's toxin is Bacillus-Calmette-Guérin (BCG); an attenuated strain of mycobacteria. Today, intra-vesical BCG instillation is an established treatment of superficial urinary bladder cancer (Morales et al., 1976) and its efficacy is dependent on the concurrent presence of $CD4^+$ and $CD8^+$ T cells (Ratliff et al., 1993). These two treatment approaches activate the immune system in an

unspecific manner, thus relying on the adaptive arm of the immune system to mount a specific response directed against the cancer cells. Another non-specific approach is administration of cytokines. The T cell growth factor IL-2 is secreted in large amounts from activated T cells and, if administered in high doses, it can cause durable remission in subsets of patients with metastatic melanoma and renal cell carcinoma (Rosenberg, 1994a). Today, insights in cellular and molecular immunology obtained during the last two decades have made more tumour-specific modalities possible. These can be broadly categorised into active and passive strategies. Active immunotherapy involves vaccination, which in the case of tumours is primarily aimed at eliciting a T cell response. In passive treatments, on the other hand, preformed effector molecules or cells are administered to the patient.

## Cancer vaccination

A vaccine consists of the antigen admixed with an adjuvant; the latter is included to increase the immunogenicity of the target. Both Coley's toxin and BCG can be viewed as adjuvants, given without simultaneous administration of the antigen. When designing a vaccine, several issues must be taken into consideration. An appropriate antigen should be chosen and administered in a form which activates the desired effector cells. Furthermore, the optimal dose, route of administration, and vaccination schedule must be determined. Vaccinology is an entire research area on its own, and beyond the scope of this thesis. The following text is limited to an overview of the different strategies employed in cancer vaccination, illustrated by examples mainly from colon cancer.

To use whole tumour cell extracts was the dominating form of antigen during the earliest years of cancer vaccination, mainly due to the lack of identified tumour antigens.

An example of this formula is OncoVAX, consisting of autologous tumour cells, admixed with BCG adjuvant (Hanna et al., 2001). Three sequential studies, including a total of 704 patients with Dukes' B or C (described in "Patients & methods") colorectal cancer have been performed. Participants were randomized to either tumour cell vaccination after curative surgery or surgery alone. No statistically significant benefit was seen in stage III patients, but a considerable improvement in overall survival in the patients with stage II disease was evident upon intention-to-treat analyses. In addition, in the group of patients with stage II disease the magnitude of the delayed type hypersensitivity skin reaction against autologous tumour cells, correlated with survival. A drawback with this approach is limited availability of autologous tumour, which may be circumvented by using allogeneic tumours or tumour cell lines. Another concern is the theoretical risk of autoimmunity through stimulation of immune responses against antigens shared with non-malignant tissues.

As an alternative, a vaccine targeting a single tumour-specific antigen may be used. If the antigen is broadly expressed across tumours of a certain origin, the vaccine may be used for groups of patients which, of course, reduce costs. Instead, there is a risk for immune escape through the selective outgrowth of antigen-loss variants. An ideal tumour antigen would therefore be a protein which is essential to the survival of the cancer cell, such as the anti-apoptotic protein Survivin (Tsuruma et al., 2004).

Whole protein vaccination with recombinant CEA has been given to 24 resected colon cancer patients, together with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant. The follow-up revealed durable antibody and T cell responses which correlated with increased survival. (Ullenhag et al., 2004). Protein vaccines are taken up and processed into peptides by the APCs of the host, and may be capable of activating both $CD4^+$ and $CD8^+$ T cells against several epitopes simultaneously.

Tumour antigen-specific vaccination can also be performed with the preprocessed antigenic peptides. As compared to whole proteins, peptides are easy to manufacture under good laboratory practice conditions. However, the MHC haplotype of the patient must be taken into consideration and to achieve a combined CD4 and CD8 T cell response class I and class II-restricted epitopes have to be combined. Another alternative to whole proteins is anti-idiotypic antibodies. These antibodies have an antigen-binding domain which mimics the surface of the antigen, which may provoke an immune response against the tumour antigen.

Vaccination with nucleic acids; DNA or mRNA encoding the tumour antigen carries several advantages. These molecules are easy and inexpensive to produce in clinical grade and results in whole protein expression. In addition, naked DNA has inherent adjuvant properties, which can be further enhanced through the insertion of the DNA sequence into a viral vector. This strategy also allows inclusion of co-stimulatory molecules, such as in the CEA-TRICOM vaccine. This is a vaccinia virus-based vector containing transgenes for CEA, together with B7-1 and two additional co-stimulatory molecules. In a phase I trial, the vaccine elicited CEA-specific immune responses in patients with advanced colon cancer, with evidence of clinical improvement in a subset of the participants (Marshall et al., 2005).

To optimise the most crucial step in vaccination, namely antigen presentation, dendritic cell-based vaccines have been developed. Autologous DC can be generated *ex vivo* from circulating monocytes and loaded with any of the antigen preparations described above. Although theoretically appealing, the general performance of DC-based vaccines have not been superior to that of other vaccine strategies (Gilboa, 2007), meaning that immunological responses are elicited in a fraction, often less than half, of patients.

With respect to clinical benefit for the vaccinated subjects, a recent overview reported a response rate of 2.6% (Rosenberg et al., 2004). Several explanations may account for this poor performance. Since many vaccine tar-

gets are non-mutated self antigens; high affinity T cells may have been deleted in the thymus or diverted into the Treg lineage (Kyewski and Klein, 2006). Accordingly, prior removal of $CD4^{+}CD25^{high}$ Tregs may improve response rates as indicated in a recent vaccination trial against renal cell carcinoma (Danull et al., 2005). Furthermore, most vaccination trials have been carried out in patients with advanced tumours, which is very different from the situation with microbial vaccinations being prophylactic. Considering the immunosuppressive state associated with late stage cancer, many patients may be unable to mount productive immune responses, no matter how elaborate the vaccination strategy is. One way to circumvent this is by passive immunotherapy, i.e., adoptive transfer of tumour-reactive lymphocytes which have been expanded *in vitro*.

## Adoptive cell transfer

This concept was pioneered by Rosenberg and colleagues, inspired by the regressions obtained with high-dose IL-2 administration to patients with malignant melanoma. In 1980, Lymphokine-activated killer (LAK) cells were described. This cell population, distinct from resting NK and T cells, was generated from PBMC which had been incubated in IL-2 for 3-4 days and was capable of killing both autologous and allogeneic cancer cells, but not normal cells. LAK cells appeared capable to induce tumour regression in 134 patients with advanced tumours of different origin. An overall response rate of 21% was reported, including both complete and partial regressions, which was superior to treatment with IL-2 alone. Almost all responders had malignant melanoma or renal cell carcinoma, but one complete and two partial regressions occurred among the 27 patients with colon cancer and an equal number in four patients with non-Hodgkin's lymphoma (Rosenberg, 1988).

The strategy evolved when it became possible to propagate tumour-infiltrating lymphocytes (TIL) from solid tumour biopsies. The difficulties in obtaining this cell population are illustrated by the success rate in the summary of the first 5 year's efforts. Following a culture period of 5-6 weeks, TILs were obtained from three out of four melanoma biopsies and expressed specific lytic activity against the autologous tumour in half of these cases. In colon carcinoma and breast cancer, success rates were higher, but instead no lytic activities were detected in any of the cultures (Yannelli, 1996). Melanoma-derived TILs were clearly distinct from LAK cells, being T lymphocytes and killing targets in an MHC-dependent manner. Because the anti-tumour activity of TILs was several orders of magnitude higher than that of LAK cells (Rosenberg, 1988), they were used in adoptive transfers to melanoma patients (Rosenberg et al., 1994b). 86 patients with metastatic malignant melanoma were treated with infusion of TILs in combination with high-dose IL-2. The objective response rate was 34%, but in the majority of cases

tumour progression ensued. Therefore, a refined protocol was tested, using cloned $CD8^+$ T cells specific for the melanoma antigen gp100. However, the cells persisted poorly after transfer and results were similarly disappointing (Dudley et al., 2001). By contrast, including lymphodepleting chemotherapy prior to cell transfer and the use of a polyclonal T cell population consisting of both $CD4^+$ and $CD8^+$ T cells, resulted in improved response rates (Dudley et al., 2005). With this strategy, transferred cells could be detected in peripheral blood several months after treatment and tumour regressions were reported in half of the patients.

Adoptive cell transfer has been attempted in other solid tumours as well. As a rule, the studies are small and results are less promising than for melanoma. Studies of adoptive cell transfer in colon cancer – the focus of study V in the present work – are summarised in Table 2. Regarding invasive urinary bladder cancer, the other main subject of the thesis; adoptive immunotherapy is essentially unexplored.

Table 2. *Adoptive immunotherapy of colon cancer*

| Patients | Cells | Outcome | Reference |
|---|---|---|---|
| 8 Dukes' D | LAK cells + IL-2 | No clinical responses. | Dillman et al., 1991 |
| 22 Dukes' D | LAK cells + IL-2 | 1 complete response | Hawkins et al., 1994 |
| 15 Dukes' D | Autologous, activated macrophages | 3 temporary disease stabilisations | Eymard et al., 1996 |
| 7 Dukes' D | PBL, co-cultured with autologous tumour cells | 1 partial response, 2 temporary disease stabilisations. | Soda et al., 1999 |
| 32 Dukes' D | CD3-expanded cells from tumour draining lymph nodes | One 80% reduction of tumour mass, 4 minor responses and 15 disease stabilisations. | Kim et al., 1999 |
| 9 Dukes' D | TIL + IL-2 | No clinical responses. | Fabbri et al., 2000 |
| 19 Dukes' D; post-surgery | TIL + IL-2 | Identical protocol, but no remaining, detectable disease. 8 patients free of recurrence at 21 months. | Fabbri et al., 2000 |
| 47 Dukes' D | TIL + IL-2 | No survival difference beteween patients receiving TIL and a control group at 1,3 or 5 years. | Gardini et al., 2004 |

Abbreviations: CC=Colon cancer, LAK=Lymphokine-activated killer, PBL=Peripheral blood leukocytes, TIL=Tumour-infiltrating lymphocytes.

## Lymph node-based immunotherapy

In agreement with the results from Rosenberg's lab (Yanelli et al., 1996); TIL-based therapies have not been successful in colon cancer (Table 2) and alternative approaches need to be explored.

As discussed previously, any immune response is, in theory, expected to be initiated in the draining lymph node. As the lymph node may be less affected by the immunosuppressive effects of the tumour; it represents an alternative cellular source for adoptive immunotherapy. In fact, the transfer of tumour immunity with lymph node cells was performed over half a century ago, in mice (Mitchison, 1955). However, the lymphatic drainage from a human solid tumour is unique and may be complicated by neo-lymphangiogeneis (Fernandez et al., 2007). The location of the truly tumour-draining lymph node is therefore not possible to predict, which impedes this approach. A solution was suggested by Triozzi et al. (1994), who injected a radio-labelled monoclonal antibody against the tumour-associated mucin TAG-72 preoperatively in colon cancer patients admitted for surgery. This enabled the detection of lymph nodes containing tumour cells or shed tumour antigen. Proliferative responses against autologous tumour cells was found in all the identified lymph nodes, whereas lymphocytes from uninvolved lymph nodes were unresponsive. Inspired by these findings the same group conducted a phase I trial, using autologous T cells from TAG-$72^{+}$ lymph nodes which were activated *in vitro* with an anti-CD3 antibody (Kim et al., 1999). The expanded cell population was adoptively transferred to patients with advanced colon cancer, with objective responses in 5 of 32 patients (Table 2).

## The sentinel node concept

Lymph nodes draining solid tumours are often the first sites for metastases to appear, an event of major prognostic importance. The sentinel node concept was originally formulated by Cabanas (1977) in penile carcinoma. It postulates that the lymphatic drainage from a tumour arrives to a primary draining, lymph node – the sentinel node, before it continues to other lymph nodes. Therefore, the histopathological status of the sentinel node can be regarded as representative for the entire lymphatic field. By peritumoural injection of a tracer substance, either a radioactive compound or a blue dye, this node can be identified intraoperatively and subjected to detailed analysis (Figure 2; page 32). The concept is widely accepted in breast cancer and melanoma, where the presence tumour cells in the sentinel node has large impact on the extent of surgery and postoperative treatment. More recently, sentinel node detection has been introduced in colon (Thörn et al., 2000), and urinary bladder cancer (Sherif et al., 2001) and has shown a great potential to improve staging (Dahl et al., 2005; Liedberg et al., 2006).

For the immunologist, sentinel node detection constitutes an excellent possibility to investigate human tumour-draining lymph nodes, independent of any presence of tumour cells. This opportunity was the actual origin of the work presented in the forthcoming pages.

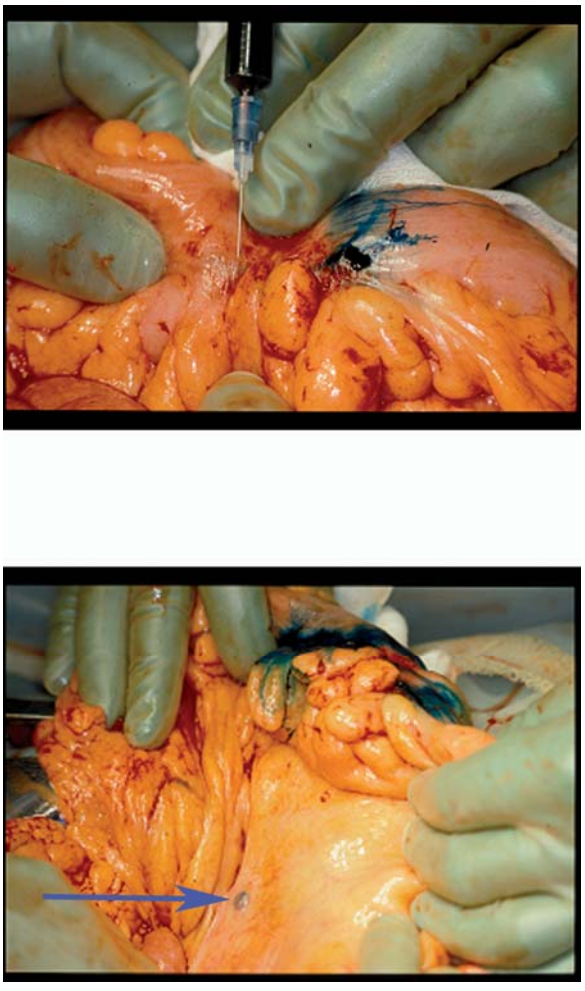

*Figure 2.* Peroperative sentinel node detection in colon cancer. Following subserosal injections of Patent blue dye around the tumour (upper panel), one or more blue-coloured lymph nodes appear in the mesentery (lower panel), usually within five minutes.

# Aims

The general aim of this thesis was to investigate the immune surveillance against solid tumours in humans, focusing on the $CD4^+$ T lymphocyte and its potential use in immunotherapy of cancer.

The specific aims were:

- To investigate the presence of anti-tumour immune responses in sentinel nodes draining human solid tumours.
- To develop a novel method for measuring T cell receptor diversity, using padlock probes and microarrays.
- To investigate the prevalence and role of regulatory T cells in urinary bladder cancer.
- To explore the possibilities of adoptive immunotherapy, using sentinel node-acquired lymphocytes in colon cancer.

# Patients & Methods

A summary of patients, materials and methods used is given below. For additional details, the reader is referred to the respective papers.

## Patients

### Pathological staging (Paper I-V)

Table 3. *Dukes' classification of colorectal cancer(Turnbull et al., 1967)*

| Dukes' stage | Description |
|---|---|
| A | Tumour limited to the bowel wall |
| B | Penetration through bowel wall |
| C | Lymph node metastasis but no signs of distant spread |
| D | Distant metastasis |

Table 4. *TNM-classification of urinary bladder caner(UICC 1997/2002)*

| Stage | Description |
|---|---|
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| Ta | Non-invasive papillary carcinoma |
| Tis | Carcinoma in situ |
| T1 | Tumour invades subepithelial connective tissue |
| T2 | Tumour invades muscle; superficially (T2a) or deep (T2b) |
| T3 | Tumour invades perivesical tissue; microscopically (T3a), or macroscopically (T3b) |
| T4 | Tumour invades adjacent urogenital organs (T4a), or pelvic/ abdominal wall (T4b) |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No lymph node metastasis |
| N1-3 | Lymph node metastasis |
| MX | Distant metastases cannot be assessed |
| M0 | No distant metastasis |

The letter "p" distinguishes the definitive classification by the pathologist post-cystectomy from the pre-operative classification based on the TUR-B specimen and clinical examination.

### Sentinel node identification (Papers I-III and V)

In colon cancer, sentinel node identification was performed peroperatively. Following mobilisation of the tumour bearing bowel segment, Patent blue dye (Guerbet, Paris) was injected in the serosa around the tumour. Usually within 5-10 minutes, tumour draining lymph nodes appeared blue. These lymph nodes were marked with sutures and dissected after complete resection of the bowel segment.

In urinary bladder cancer sentinel nodes were identified by a combination of pre- and peroperative techniques. Before operation a radioactive tracer was injected into the bladder wall. One to four hours later lymphoscintigraphy was performed or, in some cases, SPECT (Single-photon emission tomography) combined with computer tomography. Right before surgery, Patent blue dye was injected transurethrally around the tumour. Following lymph node dissection, sentinel nodes were identified by their blue colour and/or by radioactivity, as measured with a handheld γ-detection tube.

### Follow-up and response evaluation (Paper V)

The patients were followed with regular controls usually at 3, 6, 12 and 18 months post-surgery, including assessment of the general condition and measurement of carcinoembryonic antigen (CEA) in serum. Radiological examinations for liver and lung metastases were performed every six months by ultrasound, X-ray, computer tomography scan, or fluorodeoxyglucose positron emission tomography (FDG-PET).

Assessment of the clinical response was made by comparison of radiographic measurements and physical examinations before and after treatment using WHO criteria. The complete disappearance of all evaluable disease was defined as a complete response. A partial response was defined as a decrease, equal to or greater than 50%, in the sum of the products of perpendicular diameters of all measurable lesions. Stable disease was defined as no signs of clinical or radiological tumour progression.

All studies were approved by the local ethical committee and informed consent was given by the patients.

## Cell preparation (Papers I-III and V)

Single cell suspensions from tissue specimens were obtained by gentle pressure in a loose fit glass homogeniser. Peripheral blood mononuclear cells (PBMC) were purified from venous blood by density gradient centrifugation (Ficoll-paque, GE Healthcare).

In paper III, isolation of TCR $\alpha\beta^+$ or $CD4^+$ cells was performed by magnetic selection over MACS LS columns (Miltenyi Biotech) using either

FITC-conjugated primary antibodies (Becton Dickinson) and anti-FITC Microbeads (Miltenyi Biotech) or the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech), according to the manufacturer's recommendations.

## Flow cytometry (Papers I-V)

For surface staining, cells were washed in PBS supplemented with 2% FCS and 0.05% $NaN_3$ (staining buffer), followed by 30 min incubation with antibodies against markers of interest.

For intracellular stainings, cells were first treated with Cytofix/Cytoperm (Becton Dickinson) according to the manufacturer's recommendations and then kept in staining buffer supplemented with 0.3% saponin. Alternatively, permeabilisation was performed without prior fixation. In paper IV, cells were fixed and permeabilised using the FOXP3 staining buffer set (eBioscience) and stained with an anti-human FOXP3 APC antibody, according to manufacturer's protocol.

The samples were acquired on a FACS Calibur (Becton Dickinson) or a FACSAria (Becton Dickinson). Data was analysed with the CellQuest (Becton Dickinson) or FACS Diva computer software (Becton Dickinson).

## Immunological evaluation (Papers I, II and V)

Lymphocytes from sentinel- and non-sentinel lymph nodes, from the tumour and PBMC were suspended in RPMI 1640 supplemented with 10% human AB serum (Sigma), 100 units/ml of penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Autologous tumour extract was prepared by homogenisation with an Ultra Turrax in 5 volumes (w/v) 2xPBS followed by 5 min denaturation at 97°C.

For T cell proliferation assays, cells were distributed in 96-well plates at 300,000 cells per well, and stimulated with the titrated concentrations of tumour extract at 1/100 - 1/10 (v/v) for 4-8 days, or with the mitogen Concanavalin A (ConA) at 10 µg/ml for 2 days.

Cells were pulsed with 1 µCi $^3$H-thymidine (Amersham) 18 h prior to harvesting, followed by scintillation counting.

In paper I and V, stimulation of lymphocytes was performed as described for the proliferation assay and the amount of IFN-γ in the supernatant was measured by sandwich ELISA (Human IFN-γ Duoset, R&D systems).

## *Ex vivo* culture of lymphocytes (Papers II and V)

In paper II, sentinel node lymphocytes were kept in RPMI 1640 supplemented with 10% human AB serum (Sigma), 100 units/ml of penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Antigenic stimulation was provided by addition of autologous tumour extract and cultures were supplemented with recombinant interleukin-2 (Sigma) every 3-4 days.

In paper V, lymphocytes were kept in AIM V Media (Invitrogen) at a density of 4 million cells/ml supplemented with interleukin-2 (IL-2) (Proleukin®, Chiron). Three-to-four days after initiation of the cell culture autologous tumour extract was added at a dilution of 1/100 (v/v). For long-term culture the cells were retained in a cell incubator at 37˚C and 5% CO2 and IL-2 (Proleukin, Chiron) was added every 3-4 days. Restimulation was performed by addition of autologus tumour extract at a dilution of 1/100 (v/v) and autologous PBMC, irradiated with 2500 rad. The amount of IFN-γ and IL-4 secreted into the supernatant at the end of *ex vivo* culture was measured by ELISA (Human IFN-γ Duoset and Human IL-4 Duoset, R&D Systems).

## Vβ padlock probe assay (Paper III)

*Sample preparation*

All padlock probes were analysed for secondary structures using web-based softwares. Total RNA was extracted with TRIzol (Sigma-Aldrich) according to the manufacturer's protocol and cDNA was synthesised by random hexamer (Gibco BRL)-priming with the double-stranded cDNA synthesis kit (Invitrogen). Samples were purified on a G-50 Sepharose Spin Column (Amersham Bioscience) and combined with a ligation mix, containing 90 pM of each padlock probe and 1.5 U Ampligase (Epicentre). Ligation was performed at 95 °C for 5 min and then cycled four times between 46 °C for 3 h, 60 °C for 10 min, and 95 °C for 2 min. Samples were treated with Exonuclease I and III for 1 hour at 37°C, followed by 95 °C for 5 min. A polymerase chain reaction was set-up using the Platina Taq Polymerase (Invitrogen), samples were placed in a thermal cycler at 94 °C for 2 min, then cycled 25 times between 95 °C for 30 s and 56 °C for 10 s.

*Oligonucleotide arrays*

Amino-modified spotting oligonucleotides were printed in triplicate on 3D-Link slides (Motorola) as 12 subarrays using a four pin GMS 417 Microarrayer (Genetic Microsystems Inc.). Reusable silica rubber reaction chambers were prepared using an inverted microtiter plate with V-shaped wells as a

mould. Prior to adding the reaction mixtures, the rubber grid was firmly pressed against the glass surface with a plexiglas cover containing drill holes for the pipette tips. For hybridisation, 30 µl PCR reactions were combined with 20 µl of 5x standard saline citrate (SSC), 1 ml/l Triton X-100, 11 mmol/l EDTA, and 0.1 nmol/l of a hybridisation control oligonucleotide. Samples were heated at 95 °C for 1 min and immediately placed on ice. Then, 40 µl was added to the individual subarrays and hybridised at 55 °C for 1.5 h. The silica mask was removed in a 0.02x SSC washing solution, and the slide was transferred to a 0.02x SSC wash for 10 min, then dried with pressurised air. Microarrays were scanned in a Genepix 4000B (Axon Instruments), and images were analysed with QuantArray 2.0 software (GSI Lumonics). Local background on the microarray was subtracted from recorded signals, and a mean was calculated from triplicate spots. Negative results were set to zero. Sample signals ($S_{V\beta x}$) were compared with the corresponding background signals ($bc_{V\beta x;}$ padlock probe ligation reaction with no sample added) and set to zero if they were lower than $2(bc_{V\beta x})$. The representation of each Vβ subfamily was finally calculated as $S_{V\beta x} / \Sigma( S_{V\beta x}. . . S_{V\beta xn})$.

## Immunohistochemistry (Paper IV)

Serial, 4 µm-sections were cut from each paraffin block and mounted on glass slides. Following 1 hour incubation at 60°C, deparaffinisation was performed in xylene and a graded series of ethanol. Heat-based antigen retrieval was performed by microwave boiling for 20 min in Tris-EDTA (pH 9,0). Primary antibodies were a monoclonal mouse anti-human CD3 (Vector Laboratories; dilution 1:200) and a monoclonal mouse anti-human FOXP3 (Biolegend; dilution 1:100). Primary antibody dilutions were made in PBS supplemented with 1% BSA and 0,05% Tween-20 and washing steps were performed in PBS supplemented with 0,05% Tween-20. Parallel stainings without the primary antibody were performed as negative control. The ABC-ELITE (Vector Laboratories) immunohistochemical staining kit was used according to the manufacturer's recommendations for all subsequent steps. The secondary antibody was a biotinylated goat anti-mouse IgG (dilution 1:200, Vector laboratories). Stainings were developed by incubating the sections with 3, 3'-diaminobenzidine solution (Vector Laboratories) and counterstained with Mayer's haematoxylin. Quantitative evaluation was performed by two independent investigators, unaware of sample origin. The number of stained cells within tumour cell nests in ten high-power fields (x40 objective) for each antibody was counted per investigator.

## Statistical methods (Papers IV and V)

In paper IV, the data from the immunohistochemical quantification was analysed by using Student's two-tailed unpaired t-test.

In paper V, the numbers of cells received by patients with stable disease and partial responders were compared with the number of cells received by patients classified as complete responders using Wilcoxons rank sum test.

The survival time of the nine Dukes' D patients treated with immunotherapy and the control Dukes' D cohort, consisting of all patients in the Stockholm colon cancer registry with Dukes' D (n=174) operated year 2003, were plotted in an actuarial survival diagram. Survival times were compared using Log-rank analysis (Mantel cox).

# Results

## Tumour-reactive lymphocytes are present in sentinel nodes draining human solid tumours. (Paper I-II)

The common aim of the first two papers was to investigate the immunological status of the primary tumour-draining lymph nodes, i.e. sentinel nodes, in human solid tumours.

### Colon cancer

In study I, fifteen patients, with colon cancer were included. None of the patients had signs of distant metastases or lymph node involvement prior to surgery and were subjected to colectomy with peroperative sentinel node detection. One to four sentinel nodes were identified (average 2.3) and, upon dissection of the colectomy specimen, a total of five to 29 lymph nodes (average 19) were retrieved. Following histopathological evaluation, nine patients were classified as Dukes' B and five patients as Dukes' C. One patient had, in addition to one metastatic sentinel node also had liver metastases, found at surgery, and was therefore classified as Dukes' D.

Lymphocytes from the tumour, from sentinel and non-sentinel nodes and from peripheral blood were analysed by flow cytometry for the CD4 and CD8 antigens and the very early activation marker CD69. All investigated tumours were infiltrated by lymphocytes of both the CD4 and CD8 subsets. The subset composition varied between lymph nodes, but no apparent difference between sentinel and non-sentinel lymph nodes was seen with respect to any of the markers. Nor did the ratio of $CD4^+$ to $CD8^+$ cells correlate with tumour stage or the result of the proliferation assays. Proliferative responses were tested in time course $^3$H-thymidine incorporation assays using an autologous denatured tumour extract as antigen source. TIL did not proliferate in any of the investigated patients. By contrast, antigen-dependent proliferation was seen in sentinel node lymphocytes from eight of the Dukes' B patients and in one Dukes' C patient (Figure 3). Non-sentinel node lymphocytes did not proliferate, except in three Dukes' B patients. Two of these

lymph nodes were located on the predicted lymphatic drainage pathway from the tumour, suggesting that they were secondary sentinel nodes. In the third patient, all lymph nodes in the pathological specimen were enlarged and displayed histological evidence of immune reactivity. Notably, such histological findings have been correlated with improved survival (Patt et al., 1975). Tumour reactivity in the peripheral blood of colon cancer patients has been detected by Nagorsen et al. (2000). In this study, PBMC proliferated only in three cases, consistent with the dilution of tumour-antigen specific T cells in the total pool of circulating lymphocytes. Tumour-infiltrating lymphocytes and lymphocytes from two metastatic sentinel nodes were unresponsive upon stimulation with the mitogen ConA, whereas all other cell populations responded vigorously to this stimulus.

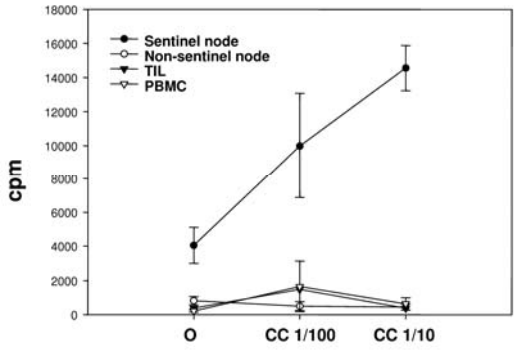


*Figure 3.* Stimulation with autologous tumour extract induces proliferation in lymphocytes from the sentinel node. Data from a patient with Dukes' B colon cancer is shown. Cell suspensions from a sentinel and a non-sentinel lymph node, tumour-infiltrating lymphocytes (TIL) and peripheral blood mononuclear cells (PBMC) were tested at 300,000 cells/well in a time-course proliferation assay with 1/100 and 1/10 dilutions (v/v) of an autologous tumour extract (CC), or medium alone (0). Proliferation was measured by scintillation counting after addition of 1μCu $^{3}$H-thymidine 18 hours before harvesting.

The IFN-γ secretory response to stimulation with autologous tumour extract was investigated and generally correlated closely with the proliferative responses (Figure 4; page 42). In one Dukes' B patient, however, TIL secreted IFN-γ despite absent proliferation and so did PBMC from this and another Dukes' B patient. Possibly, these cells were at a more advanced stage of effector cell differentiation than those present in the sentinel lymph nodes

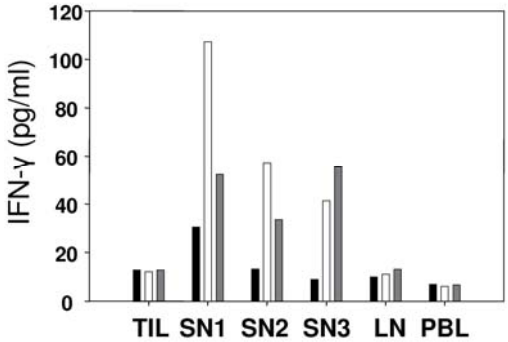


*Figure 4.* Secretion of IFN-γ in response to the autologous tumour extract. Lymphocytes from sentinel nodes (SN1-3), from the tumour (TIL) or peripheral blood (PBL), were cultured in medium alone (black bars), or stimulated with tumour extract diluted 1/100 (open bars) or 1/10 (grey bars) (v/v). The amount of IFN-γ present in the supernatants was measured with a sandwich ELISA. The data shown in the figure is from a patient with Dukes' B colon cancer and the samples were collected after 5 days of culture.

## Urinary bladder cancer

In study II, fourteen patients, admitted for cystectomy due to urinary bladder cancer, were investigated. None of them had known distant metastases and they had not received neoadjuvant therapy prior to surgery. Two to eight sentinel nodes (average 3.2) were identified. In two patients no sentinel nodes were detected, in one case due to extensive fibrosis in the true pelvis and in the other due to a locally advanced primary tumour. Seven patients had lymph node positive disease upon pathological assessment. Single cell suspensions from the tumour, lymph nodes and peripheral blood were analysed by flow cytometry including the markers CD4, CD8, CD69 and the lymph node homing receptor CD62L, which is downregulated upon activation. As in colon cancer, both $CD4^+$ and $CD8^+$ cells were present in the tumour. In the lymph node samples the combined pattern of the activation markers CD69 and CD62L was similar in sentinel and non-sentinel lymph nodes and so was the relation between the CD4 and CD8 subsets. Intracellular staining against the urothelial cell marker cytokeratin-20 was performed to address the presence of tumour cells within the lymph nodes. This assay was able to detect tumour cells in metastatic lymph nodes, in accordance with the pathological verdict.

Immunological reactivity of the cells against an autologous denatured tumour extract was tested in time course $^3$H-thymidine incorporation assays. No responses were detected in TIL, whereas antigen-dependent proliferation

was detected in sentinel nodes from five of the patients. Interestingly, in two cases proliferation was confined to the only investigated lymph nodes containing tumour cell deposits. This contrasts with the results in study I, where patients with lymph node metastases displayed weaker or absent proliferation. In two patients, non-sentinel nodes proliferated in two patients. In one of these, there was simultaneous proliferation in the sentinel node, whereas in the other case, no sentinel nodes were detected. In this and another patient, proliferation was seen in PBMC. These responses were less vigorous and delayed as compared with those in the corresponding lymph nodes. TIL did not respond to stimulation with ConA, but the mitogen gave rise to strong proliferation in all lymph node cells and in PBMC.

From three patients, sentinel node lymphocytes were obtained in sufficient numbers for *in vitro* culture, with the autologous tumour extract as sole antigen source. Cells were kept in culture for several weeks, despite the fact that one of the patients was non-responsive in the proliferation assay. During cultures, $CD4^+$ lymphocytes were enriched. Furthermore, the expression of the activation marker CD69 increased and CD62L was downregulated.

*Conclusion*

Sentinel nodes draining colon and urinary bladder cancers harbour tumour-reactive lymphocytes, which are capable of proliferation and IFN-γ secretion directly *ex vivo*, indicating a Th1 response. By addition of tumour antigen and IL-2, lymphocytes from sentinel nodes can be kept in culture for several weeks, gradually developing a more activated phenotype. These results indicate that the sentinel node-acquired lymphocytes may be suitable for adoptive tumour immunotherapy. This possibility was explored in study V of this thesis.

## Padlock probe-based detection of common clonal expansions in tumour-infiltrating lymphocytes and sentinel lymph nodes (Paper III)

The immunological responses detected in study I and II suggested a clonal expansion of tumour-reactive lymphocytes. This may be detected as an altered pattern of TCR Vβ gene usage in a cell population. However, available methods for TCR Vβ repertoire analysis are labour-intensive and costly. Therefore, a novel method for analysing the Vβ gene distribution using padlock probes and a microarray readout was developed. Padlock probes are linear oligonucleotides which 5' and 3' ends are designed to hybridise next to each other on a target sequence (Nilsson et al., 1994). A DNA ligase can join the ends, thereby converting the probes to circular molecules, which embodies the presence of the target in a sample. The assay is de-

scribed in figure 5. Padlock probes for each of 24 Vβ subfamilies were constructed. The segment connecting the target complementary arms contained sequences recognised by a PCR primer pair common for all probes, and a Vβ family-specific tag sequence. Following incubation of the mixture of all padlock probes with a cDNA sample and a ligase, circularised probes were amplified by PCR, incorporating the flurophore TAMRA. The PCR product was then hybridised to a microarray, spotted with oligonucleotides complementary to the tag sequences, thus providing a simultaneous readout of the entire Vβ repertoire.

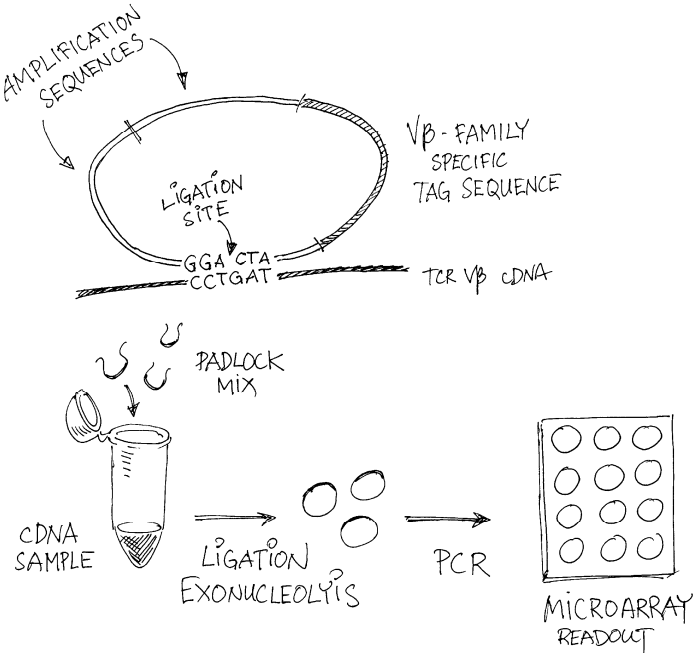


*Figure 5.* Outline of the Vβ padlock assay. Upper panel: A padlock probe binding to its target. Lower panel: Upon analysis, all padlock probes are combined with the sample in a single tube, following ligation and digestion of linear probes, the circularised probes are amplified in a PCR with a fluorescently labelled primer pair. The signals from the different probes are spatially separated by hybridising the PCR product to a microarray chip by means of the Vβ-family specific tag sequences.

First, the method was used to assay a pool of synthetic oligonucleotide targets several times. These data were used to determine thresholds for experimental variation of each individual Vβ subfamily. Repeated analysis of the same samples gave very similar results, demonstrating the robustness of the method.

Second, the assay was validated on cDNA from PBMC which had been stimulated with the superantigen Staphylococcal enterotoxin B (SEB). Superantigens stimulate all T cells expressing certain Vβ genes, resulting in

activation of a large fraction of the T cell population (Li et al., 1999). The Vβ repertoire of the cells was investigated by repetitive measurements during 8 days post-stimulation, both at the protein level by flow cytometry and with the padlock probe assay. By flow cytometry a decrease in the percentage of cells in the $CD3^+$ population expressing Vβ 3, Vβ 12, Vβ 14, and Vβ 17 at 48 and 72 h was shown, consistent with TCR downregulation upon antigen binding (Niedergang et al., 1995). This was followed by a correspondingly dramatic increase in the very same Vβ families at day 6. Measurements of Vβ distributions at the RNA level with the set of padlock probes revealed increased representations of the same Vβ families already by 48 h, which were maintained throughout the culture period. These results are in accordance with the literature (Choi et al., 1989; Lennon et al., 2000) and the assay is thus able to accurately measure the transcriptional status of a cell population.

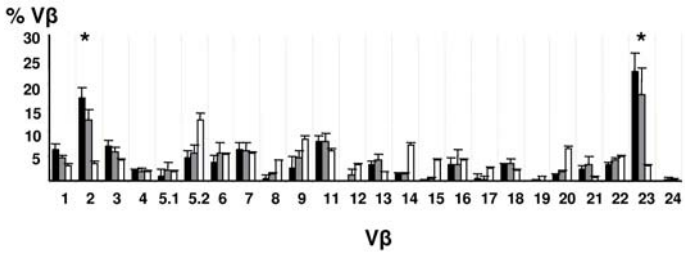


*Figure 6.* Padlock-based measurement of Vβ repertoires from TIL (black bars), sentinel node (grey bars) and a non-sentinel node, obtained from a patient with squamous cell carcinoma of the bladder. Asterisks denote common Vβ expansions in TIL and the sentinel node (Reprinted with permission by AACC).

The assay was then applied to clinical samples from cancer patients. In samples from a patient with a squamous cell carcinoma of the bladder, total $TCR\alpha\beta^+$ T cells was analysed; predominant Vβ 2 and Vβ 23 representation was found among TIL and in a sentinel lymph node, as compared with a non-sentinel lymph node (Figure 6). Another patient, with an adenocarcinoma confined to the bladder wall, displayed Vβ profiles of $CD4^+$ TIL and $CD4^+$ sentinel node-acquired T cells, dominated by Vβ 13. By contrast, Vβ 13 was undetectable in the expression profile in a non-sentinel lymph node from this patient. Finally, lymph nodes from two patients with malignant melanoma, from whom the primary tumours had been previously removed, were investigated. Divergent expression profile of sentinel and non-sentinel lymph nodes were seen in both patients, whereas the two sentinel nodes obtained from one of the patients displayed similar Vβ profiles.

*Conclusion*

The padlock probe method allows sensitive measurement of the expressed Vβ repertoire in a cell population. The assay principle comprises a few reaction steps in a single vessel and is therefore suitable for processing large numbers of samples. Alterations in Vβ family distributions are easily detected when compared with a reference sample, such as unstimulated cells or PBMC. The detection of common Vβ expansions in TIL and sentinel nodes from patients with urinary bladder tumours demonstrates the method's usefulness in a clinical setting. In addition, the findings confirm the presence of expanded tumour antigen-specific T-cells in sentinel nodes which are capable of infiltrating the tumour tissue.

## FOXP3$^+$ regulatory T cells infiltrate urinary bladder cancer (Paper IV)

The absent proliferation in TIL observed in study I and II, despite simultaneous proliferation in the sentinel node suggested an active immunosuppressive mechanism, such as the presence of regulatory T cells. In study IV, the presence and impact of these cells in patients with urinary bladder cancer was investigated by use of antibodies against FOXP3; the hallmark transcription factor of the Treg subset. By flow cytometry, CD4$^+$ FOXP3$^+$ cells, with a surface phenotype consistent with Tregs were identified in both tumour tissue, in normal bladder mucosa and in regional lymph nodes. We found that 15.0% of CD4$^+$ TIL expressed FOXP3, as compared with 14.1% in the urothelium, 11.3% - 14.6% in the lymph nodes and 7.2% in peripheral blood. In order to evaluate the clinical significance of Treg presence in the tumour microenvironment, we turned to immunohistochemistry of paraffin-embedded tumour specimens.

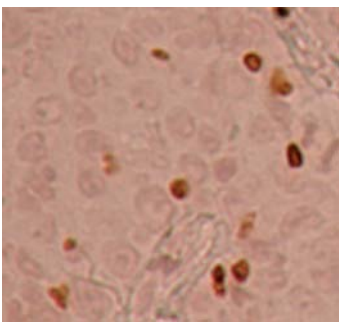

*Figure 7.* FOXP3$^+$ lymphocytes infiltrating a urinary bladder cancer

Twenty patients with urothelial cancer undergoing surgery during 1998-2000 were investigated by stainings against CD3 and FOXP3 on adjacent tissue sections. Both CD3$^+$ lymphocytes and FOXP3$^+$ Tregs were present in all the investigated samples, but to a varying extent. Stainings were quantified by counting the number of stained cells under the microscope by two independent investigators. To this end, we decided to focus on lymphocytes in close proximity to tumour cells. The ratio of Tregs to total T cell count was calculated

by dividing the mean number of $FOXP3^+$ cells in twenty high-power fields to the corresponding number of $CD3^+$ cells. Three patients were excluded from the subsequent survival analysis; one patient had a tumour located in the distal part of the urethra, which implies a worse prognosis than bladder tumours. In two other patients, no cystectomy had been performed due to advanced primary tumours. Of the remaining 17 patients, seven patients were alive at 7 years post-cystectomy. These patients had a significantly lower FOXP3/CD3 ratio than the ten patients who had died during follow-up ($p=0.05$). The absolute numbers of $FOXP3^+$ cells or $CD3^+$ cells were not significantly associated with survival, and no significant correlation was seen between tumour stage and any of the staining variables.

*Conclusion:*

Naturally occurring $FOXP3^+$ Tregs are present within human urinary bladder tumours. Their relative frequency in total $CD3^+$ TIL appear to influence long-term survival. This, as well as other regulatory cell subsets should be investigated in a larger cohort of patients.

## Sentinel node $CD4^+$ Th1 cells induce tumour regression in humans (Paper V)

The combined findings from papers I-III indicated the presence of an *in vivo* expanded population of tumour-reactive T cells in the sentinel node. In paper V, the possibility of using this cell population in adoptive immunotherapy was investigated. Sixteen patients with colon cancer were included in the study. Five of the patients had Dukes' B tumours, two cases were Dukes' C, and nine were Dukes' D. In three patients, the primary tumour had been resected on a previous occasion and they now underwent surgery due to recurrent disease, abdominally or in the liver.

*Expansion of sentinel node- acquired lymphocytes*

Sentinel nodes were identified in all patients. The sentinel node acquired lymphocytes were cultured in serum-free medium and stimulated with autologous tumour extract to ensure clonal expansion of tumour-reactive cells. A low dose of interleukin-2 was added to overcome immunosuppression and promote expansion of antigen-activated cells (Figure 8; page 48).

The cells were held in culture for approximately 5 weeks (range 23-58 days). After about 3 weeks, cultures were restimulated with autologous tumour antigen together with irradiated, PBMC, acting as antigen-presenting cells.

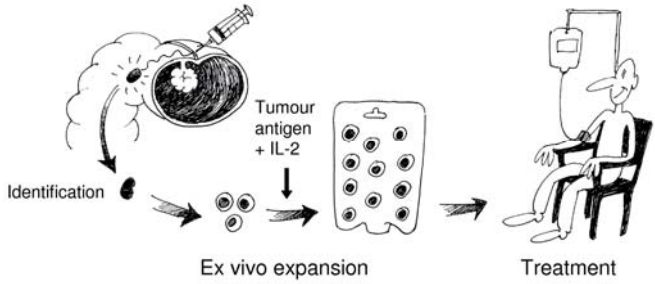


*Figure 8.* Sentinel node-based immunotherapy. Following peroperative sentinel node identification, lymphocytes are collected and put in *ex vivo* culture. Selective growth of tumour-reactive T cells is promoted by stimulation with autologous tumour extract and supplementation with the T-cell growth factor IL-2. After approximately four to five weeks the clonally expanded lymphocytes are given back to the patient as a transfusion.

The average number of sentinel node-acquired lymphocytes at the start was 115.2 million cells (range 3.6-509). Cells were monitored and characterised by flow cytometry during the expansion period. The starting CD4/CD8 ratio was 4.8 on average. Apart from T lymphocytes, B lymphocytes and a low number of natural killer (NK) cells were present in sentinel nodes. Initially, the total number of cells decreased. B lymphocytes disappeared almost completely and the number of $CD8^+$ cytotoxic T cells diminished, resulting in an enrichment of $CD4^+$ lymphocytes. This is consistent with the mode of antigenic stimulation of the cultures, since processing of exogenous intact proteins via endocytosis is expected to favour presentation on MHC class-II molecules. A clonal expansion of $CD4^+$ T cells was verified by comparing Vβ repertoires at the start and end of the cultures. Interestingly, in some cases a substantial number of $CD8^+$ cytotoxic T cells developed against extracellular antigens, most likely through cross-presentation.

Measurement of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 in cell culture supernatants, revealed high levels of IFN-γ (mean 956 pg/mL) but only small amounts of IL-4 (mean 11 pg/mL), indicating that the expanded T lymphocytes were functional and Th1 responsive. On average 71 million lymphocytes, with a mean CD4/CD8 ratio of 86.6, were re-transfused to each patient.

*Patient outcome*

No toxic side-effects were observed which could be related to the cell transfusion. The patients were followed for 29 months on average (range 6-41), with repeated clinical and radiological evaluations. Four Dukes' D patients had complete responses (CR) with no detectable remaining tumour and two patients, also with Dukes' D tumours, displayed partial responses (PR) with

diminished tumour burden. The remaining ten patients had extended periods of stable disease (SD) after the cell transfusion.

The results in the patients with distant metastases are perhaps the most interesting. The four Dukes' D patients who displayed CR were all transfused with high numbers of lymphocytes, whereas the Dukes' D patients responding with SD and PR received significantly fewer cells ($p<0.05$), indicating a dose response. The cumulative survival of our 9 treated Dukes' D patients was compared with all Dukes' D cases in the Stockholm region during the year of 2003 (n=174). The survival plot (figure 9) shows a significantly increased survival in the immunotherapy treated group (P=0.048), with an average survival of 2.6 years compared to 0.8 years for the control group.

The five Dukes' B patients all displayed high-risk tumour characteristics such as ulcerations and growth of tumour cells along nerves and in vessels. One Dukes' B patient developed liver recurrences, which responded to immunotherapy with regression of metastases. He is now free of recurrences and classified as having stable disease. The other Dukes' B patients and the two Dukes' C patients have had no signs of recurrences and were thus classified as stable disease.

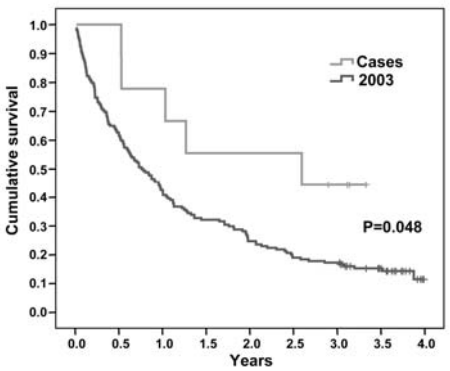


*Figure 9.* Significantly increased survival in the 9 immunotherapy-treated Dukes' D patients compared with a conventionally treated control group (n=174).

Since this was the first study of sentinel node-based immunotherapy, some patients also had chemotherapy. Of all sixteen patients, seven were treated with chemotherapy after the adoptive cell transfer, but no regular chemotherapy schedules were applied. Among the Dukes' D patients, only one received full treatment, while three patients received no chemotherapy at all and three patients had incomplete treatments due to adverse effects and/or limited compliance. The two remaining Dukes' D patients received chemotherapy after primary surgery, but developed metastases at least one year later, and at that time had sentinel node based immunotherapy, without re-

ceiving any further chemotherapy. Thus, the clinical results and increased survival among immunotherapy treated Dukes' D patients cannot be explained by the chemotherapy.

*Conclusion:*

The study demonstrates that freshly isolated sentinel node-acquired lymphocytes can be expanded against autologous tumour homogenate and retransfused to the patient without complications, as adoptive immunotherapy. Further, the results indicate that this cell population is capable of inducing tumour regression in patients with metastatic colon cancer. Considering the results in study II, adoptive immunotherapy with sentinel node-acquired lymphocytes may also be applicable in urinary bladder cancer and, possibly, other solid malignancies.

# General discussion

In essence, this thesis describes detection of tumour reactive T cells in sentinel lymph nodes, draining human colon and urinary bladder cancer, and their subsequent application in clinical immunotherapy. Tumour immunology is a rapidly expanding research area and since the work on paper I and II began in 2002, much has happened. In particular, the importance of immune escape mechanisms has become evident and tumour immunotherapy has made a definitive move from bench to bedside. The findings therefore need to be put in a larger perspective.

In the literature, there are several reports on immune suppression of tumour-draining lymph nodes, especially in the context of malignant melanoma and breast cancer (Shu et al., 2006). However, with respect to other malignancies the picture is more heterogeneous. Similar to our data, reactivity against the autologous tumour have been detected in tumours of the stomach (Stulle et al., 1994), lung and nasopharynx (Vose et al., 1977), whereas TIL responded poorly or not at all. With respect to colon cancer, Pihl et al. (1976) detected tumour-specific cytotoxicity in one of four mesenteric lymph nodes located in proximity to the tumour. Notably, no specific method was used to identify tumour-draining nodes in this study. By contrast, Triozzi et al. (1994) found proliferative responses in all tumour-draining lymph nodes, as identified with a tumour-antigen specific antibody, but not in other mesenteric nodes. Several factors may contribute to the discrepancies between tumour types, including anatomical and functional differences in lymphatic drainage and immune cell composition between tissues, as well as inherent tumour characteristics such as stage, antigenicity and acquired immune escape mechanisms. Clearly, findings in melanoma – the favourite malignancy in tumour immunology – should not be generalised to other tumours too easily.

The proliferative responses detected in study I (colon cancer) and II (urinary bladder cancer) were interpreted as the result of *in vivo* expansions of tumour-reactive T cells in the sentinel nodes, as supported by the findings in study III of common clonal Vβ expansions in TIL and sentinel nodes. The presence of active, tumour-induced immunosuppression was suggested by the dampened or absent responses in TIL and metastatic lymph nodes, but the findings contradict immunological ignorance of the tumour. In addition, the anergic phenotype of lymphocytes could be overcome by tumour-antigen

stimulation in the presence of IL-2 and with these premises; the idea of sentinel-node based immunotherapy arose.

The concept was evaluated in a phase I study, including 16 patients with advanced or high-risk colon cancer (study V). The main findings; lack of toxicity and objective tumour regression in 6 of 9 Dukes' D patients is encouraging and the treatment should be evaluated in future clinical trials.

To date, the dominating approach in adoptive immunotherapy has been anti-CD3/anti-CD28 expanded TIL, mainly composed of $CD8^+$ T cells, given in combination with high doses of IL-2 (Dudley et al., 2005). We expanded sentinel node-acquired lymphocytes by stimulation with tumour antigen and autologous APC, resulting in a mixed population with a predominance of $CD4^+$ Th1 cells. T helper cells in adoptive immunotherapy have long been considered dispensable or, at best, supportive for the cytotoxic $CD8^+$ T cell response. However, recent experimental data have demonstrated both their ability to reject MHC class II negative tumours all by themselves as well as their synergistic co-operation with $CD8^+$ T cells (Wang et al., 2007). Furthermore, in a large retrospective study on histopathological data and outcome in colon cancer a correlation between a Th1 immune response and prolonged survival has been described (Pagès et al., 2005). An additional benefit of the cytokines produced by activated $CD4^+$ T cells is that the protocol becomes independent of supportive, exogenous IL-2 administration to the patient, which is a highly toxic treatment.

Our protocol did not employ chemotherapy prior to cell transfer, which has been used by others (Dudley et al., 2005). As the size of the T cell compartment is tightly regulated, the motive behind lymphodepleting preconditioning is to provide "space" for the adoptive transfer and also to eliminate regulatory T cells. Following this treatment, *in vivo* expansion of the transferred cells and improved long-term persistence was reported. However, chemotherapy adds toxicity to a treatment which is intended for elderly patients, and alternative approaches deserve to be explored. For example, the cytokines involved in T cell homeostasis are beginning to be elucidated and enhancing the survival of specific, cellular subsets by co-administration of these mediators is appealing.

During the past five years the interest in $CD4^+CD25^{high}$ Tregs has exploded and their suppressive role in malignancy has been recognised. The relative importance of Tregs in a certain tumour is likely dependent on their antigen specificity. Our results in study IV suggest that they contribute to immune escape in urinary bladder cancer and others have analysed their presence in colon cancer patients (Loddenkemper et al., 2006). Selective depletion, or targeting of their inhibitory functions is another attractive approach to improve treatment results.

The combination of immunotherapy with traditional oncological treatments will likely have synergistic effects, provided that dosage and timing is optimised. Radiotherapy, for instance, increase both MHC class I expression

and the intracellular pool of peptides available for presentation (Reits et al., 2006), whereas chemotherapeutic regimens incorporating cyclophosphamide may preferentially target Tregs (Ghiringhelli et al., 2004).

Adoptive transfer of PBMC that had been transduced with a retrovirus encoding a tumour antigen-specific TCR, in this case MART-1, was recently reported (Morgan et al., 2006). Although the response rate was comparatively low (13%), it was argued that genetically engineered lymphocytes may constitute the next generation of immunotherapy. However, this approach is limited to shared tumour antigens, mostly being non-mutated proteins. In my view, the most promising targets for tumour immunotherapy are the private, mutated antigens. These altered proteins have not been exposed to the immune system during development and high-avidity TCRs may thus remain in the mature repertoire It has been estimated that approximately 90 mutations in a colon cancer give rise to altered amino acids (Sjoblom et al., 2006) and consequently, there should be plenty of private antigens for the immune system to recognise. As suggested by TCR repertoire analysis (study III), the natural immune response against the tumour is likely oligoclonal. Preferably, an immunotherapeutic regimen should also target multiple epitopes to minimise the risk for immune escape by antigen loss.

During the past decades, tumour immunology has moved from the criticised immunosurveillance hypothesis to actually curing patients with advanced cancer. It is easy to identify large gaps in our understanding of the interactions between tumours and the immune system. However, through joint efforts in oncology, tumour biology and immunology, these gaps can be filled and immunotherapy of cancer incorporated into clinical practice within the near future.

# Populärvetenskaplig sammanfattning på svenska

Immunförsvarets främsta funktion är att skydda människokroppen från sjukdomsalstrande mikroorganismer. För att utföra sin uppgift på ett säkert sätt, måste immunsystemets celler klara av att skilja på själv, dvs. kroppsegen vävnad och icke-själv – inkräktaren.

Cancerceller har förändringar i arvsmassan (mutationer) som ger dem överlevnadsfördelar framför andra celler i kroppen. Maligna tumörer växer dessutom utanför sin ursprungsvävnad och avsätter dottertumörer (metastaser). Det är dessa som oftast dödar patienten och vid metastaserad cancersjukdom är prognosen ytterst dålig, trots dagens behandlingsmöjligheter i form av kirurgi, strålning och cellgifter.

Cancercellens mutationer ger dock upphov till förändrade proteiner och dessa kan uppfattas som icke-själv av immunsystemet. I djurmodeller har det visats att vita blodkroppar (lymfocyter) av en viss typ, så kallade T-celler är viktiga vid försvaret mot tumörer. Lymfocyterna cirkulerar i blodbanan, men lämnar denna med jämna mellanrum för att stanna upp i lymfkörtlar. I en lymfkörtel får T-cellen tillfälle att möta proteiner som transporterats dit med lymfkärl från omkringliggande vävnader. Ett främmande protein; ett *antigen*, gör att en del T-celler aktiveras och börjar dela sig, förutsatt att T-cellen även får stimulerande signaler från omkringliggande celler i lymfkörteln. Den intensiva celldelningen ger upphov till en *klon* av identiska dotterceller som lämnar lymfkörteln för att leta reda på inkräktaren.

Cancer sprids ofta via lymfkärlen och förekomst av metastaser i lymfkörtlarna är avgörande för prognos och behandling. För att förbättra lymfkörtelbedömningen har man utvecklat metoder för att hitta den körtel som först tar emot lymfdränaget från en tumör: portvaktskörteln, även kallad sentinel node. En blå färg, alternativt ett svagt radioaktivt ämne, sprutas kring tumören. Markören följer sedan med lymfflödet till sentinel node som därigenom identifieras, antingen genom sin färg eller genom sin radioaktivitet.

I den här avhandlingen användes sentinel node-detektion för att studera immunsvaret mot två vanliga cancerformer: tjocktarms- och urinblåsecancer. Vid operationen då primärtumören togs bort erhölls vävnadsbitar dels från själva tumören och dels från en eller flera lymfkörtlar. När lymfocyter från sentinel node stimulerades med ett extrakt från patientens egen tumör började cellerna i många fall dela sig samt utsöndra interferon-gamma, ett ämne

som tidigare visats vara av betydelse för immunologiskt skydd mot tumörer. Som jämförelse undersöktes lymfocyter som infiltrerade tumören; dessa svarade inte alls på stimuleringen, något som tyder på att tumören aktivt hämmar immunsvaret. Vid tjocktarmscaner tenderade även lymfocyter från metastatiska sentinel nodes att svara dåligt eller inte alls. När dessa sentinel node-celler sattes i cellodling och stimulerades upprepade gånger med tumörextrakt och interleukin-2 – en tillväxtfaktor för T-celler, började de emellertid att dela sig.

T-celler känner igen ett antigen med en speciell ytstruktur som kallas T-cellsreceptor, vars exakta uppbyggnad skiljer sig mellan olika T-celler. Vid en aktivering och klonal expansion av T-celler, kommer vissa T-cellsreceptorstrukturer blir mer frekventa, vilket kan användas för att indirekt påvisa en antigen-specifik celldelning. När T-cellreceptorprofiler från tumörinfiltrerande lymfocyter och i sentinel nodes undersöktes på gennivå sågs likartade klonala expansioner, vilket tyder på att de reagerat på samma antigen. Tillsammans med stimuleringsförsöken med tumörextrakt pekar detta på att en naturlig expansion av tumörreaktiva T-celler har ägt rum i sentinel nodes.

Hämningen av tumörinfiltrerande lymfocyter fick oss att undersöka förekomsten av regulatoriska T-celler i tumörvävnaden. Dessa T-celler hämmar aktiveringen av andra lymfocyter och kännetecknas av proteinet FOXP3. Paraffininbäddad tumörvävnad från 20 patienter med muskelinvasiv urinblåsecancer diagnostiserade 1998-2000 undersöktes med antikroppar mot FOXP3 samt mot CD3 – en markör gemensam för alla T-celler. Antalet regulatoriska T-celler i förhållande till totalmängden T-celler i tumören var lägre hos patienter som fortfarande levde 7 år efter det att primärtumören opererats bort. Detta indikerar att tumörinfiltrerande regulatoriska T-celler kan påverka prognosen negativt för patienter med urinblåsecancer.

Slutligen undersöktes möjligheten att använda tumörreaktiva lymfocyter från sentinel nodes i behandlingssyfte. Sexton patienter med tjocktarmscancer, varav nio med fjärrmetastaser, rekryterades till en pilotstudie. I samband med operation, då primärtumör respektive metastaser togs bort, identifierades sentinel nodes. Lymfocyter från dessa renades fram och placerades i cellodling med tillsats av interleukin-2. För att selektivt expandera tumörspecifika lymfocyter stimulerades cellerna med ett extrakt från patientens egen tumör. Efter cirka 5 veckor i cellkultur gavs de expanderade lymfocyterna, i genomsnitt 71 miljoner till antalet, tillbaka till patienten som en transfusion. Behandlingen gavs i samtliga fall utan biverkningar och patienterna följdes med regelbundna kontroller i medeltal 30 månader därefter. Fyra av patienterna med spridd cancer uppvisade så kallat komplett respons, det vill säga; ingen detekterbar tumörväxt fanns kvar. I två fall minskade befintliga tumörhärdar i storlek. Vid en jämförelse med patienter med spridd tjocktarmscancer som erhållit konventionell behandling, hade de nio patien-

ter som ingick i studien en signifikant längre överlevnad (2,6 jämfört med 0,8 år).

Sammanfattningsvis: en naturlig klonal expansion av tumörreaktiva T-celler påträffades i sentinel nodes vid tjocktarms- och urinblåsecancer. En bidragande orsak till att cancern inte bekämpas effektivt av dessa lymfocyter kan vara en hög närvaro av regulatoriska T-celler i tumörvävnaden. Slutligen visas det att immunterapi med T-celler från sentinel node kan minska tumörbördan och öka överlevnaden vid avancerad tjocktarmscancer. Denna behandlingsstrategi kan och bör även undersökas vid andra tumörsjukdomar.

# Acknowledgements

I am grateful to all the people who have supported me in during these years, with special thanks to:

*Ola Winqvist*, my supervisor, for being the best possible tutor I can imagine. Since our first encounter at Eriksbergs vårdcentral, you have thoroughly initiated me into the fascinating world of science; from the art of pipetting to more theoretical issues. Somehow, you have managed to always have time – possibly because you have not, yet, discovered the word "no". In addition, you are a great person and friend. I still haven't ceased to be impressed by your capacity and your multi-faceted talents.

*Magnus Thörn*, my co-supervisor, for your deep commitment to our research, for encouragement and 24-hour delivery service.

*Ulla Lindqvist*, the head of the Department of Medical Sciences, for supporting my research.

*Mona Karlsson*, my friend and co-author. Despite (or because of…?) our different personalities I couldn't have had a better sparring-partner. Thanks for your enthusiasm, spontaneity, trust and for putting up with my varying degree of social competence during irregular working hours.

*Signe Hässler*, friend and colleague: your passion for science has been a true source of inspiration, even though your commitment sometimes made me feel a little lazy… Thanks for your care and for generously sharing your thoughts, both on research and in other matters.

*Amir Sherif*, colleague and appreciated research partner, sometimes even more interested in my work than myself! Thanks for your enthusiasm and for putting up with my nagging about patient data.

All the members of *the Immunotherapy group* (I guess the former name: "Ola and the youngsters", has expired): *Emma Lundgren*, for good spirits and for sharing my frustration over untidy lab-benches and delayed trains; *Peter Jansson*, for enjoyable collaboration and imaginative (but quite demanding) scientific discussions; *Petra Jones*, for being such a nice and gen-

tle person; *Maria Lampinen* – for pleasant company in Uppsala, Stockholm and Belgium and for your down-to-earth approach to research; *Malin Blennow* – for your bubbling enthusiasm and crucial support on paper IV; *Mikael Eberhardson*, for for providing us with a breath of aristocracy every now and then; *Sara Lind* – for contributing to the good atmosphere in the group; *Patrik Larsson* and *Johan Brännström* – for the company during the years in Uppsala. Thank you all for creating the perfect workplace!

*Johan Banér*, my co-author on paper III, for fruitful collaboration and for providing insights into molecular biology at the pico-molar level.

*Anna Tolf*, for taking active interest in our work and for invaluable assistance on paper IV

*Kjell Dahl*, co-author on paper I and V, for engaging in our research and adding surgical competence to the team.

All the people at CKMF for company and support during these years.

Professor *Olle Kämpe* and all the members of *Lab 21* (both former and present) for welcoming me to the department and inviting me to the Monday lunch meetings.

*Mohammad Alimohammadi*, colleague in many aspects, for sharing both scientific and clinical concerns.

*Thomas Lorant*, colleague and neighbour in FoA III, for immunological discussions and for persuading me to play a round of golf now and then.

*The Molecular medicine group(Lab 20)*, for helpful technical assistance with microarray scanning.

All my "new" colleagues at *L2:04*, for welcoming our group from Norrland in the first place, for being so helpful and for creating a pleasant atmosphere to work in.

*Birgitta Sembrant*, *Lillebil Andersson*, and *Anne Rasikari* for your kind help with administrative matters.

*Joachim Lundahl*, head of Transfusion medicine and Clinical immunology, for approval of my research and providing time off to complete this thesis.

*Johanna Strindberg*, my tutor in transfusion medicine, for taking great care of me during my first months at the blood bank.

All of my colleagues in Huddinge, for making me feel welcome, and for generous advice in transfusional issues.

*Jarmila Zettergren* and the personnel at Tierps vårdcentral for a fun and instructive time and for allowing me to feel like a "real" doctor.

Friends (not included in the categories above) – thank you all for remaining in my circle of acquaintance despite my irregular contact habits:

*Axel, Anna, Lina* and *Lovisa Nordenskjöld*, for your hospitality, for shared memorable moments and for trying to make me understand the importance of good food.

*David, Liz, Sam*, and *Isak Bakall*, and for caring and enjoyable company.

*Thomas, Hedvig* and *Vilgot Tängdén*, for good times spent together and for remaining resident in Uppsala.

*Lisa Helldahl*, for appreciated long-distance friendship and stimulating exchange of thoughts and literature.

*My Family*, for providing an invaluable context to my life.

*My Mother*, for your never-ending love and support and for providing me with continuous updates from contemporary primary care.

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