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ON LYMPH NODE BASED IMMUNOTHERAPY OF CANCER

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ON LYMPH NODE BASED IMMUNOTHERAPY OF
CANCER
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To my beloved family

ABSTRACT

Cancer Immunotherapy based on one's own immune system to fight cancer is emerging as an important treatment option for advanced stage diseases. The earliest attempts of cancer therapy based on the provocation of the immune system with infectious agents can be traced back to the 18th century, and the field of immunotherapy has since developed into three main groups: cellular-, antibody- and cytokine- therapies. Recently, especially the treatment with so called checkpoint blocking antibodies has led to a breakthrough in the treatment of some cancers, and at the time of writing this thesis the Nobel Prize in medicine is to be awarded to the discoverers of the two first such checkpoint pathways targeted with successful, approved drugs. These agents however, seems not work if there is not a weak suppressed immune response already in the patient and alternative strategies are needed to help the patients where checkpoint blockade is unsuccessful. One such immunotherapy that has proven successful is adoptive transfer of tumor specific T-cells grown in the lab to the patient. Previous studies in our research group found that sentinel lymph nodes, which are the lymph nodes first draining the tumor, may be sites of specific T-cell responses to the tumor since antigens from the tumor are presented there by professional antigen presenting cells. Due to the fact that these lymph nodes are also the first ones to be colonized by metastasizing tumor cells from the primary tumor, the histopathological status of these tumor draining lymph nodes is essential for staging and prognosis, and this analysis usually involves destructive processing of the lymph node tissue. As the lymph node T-cells are a potential source for tumor-specific T-cell therapy of cancer, it is essential to find a method of metastasis detection that does not require destruction of the whole lymph node tissue. Thus our lab has developed a method to dissociate the cells of the lymph node for flow cytometric analysis based on markers found on tumor cells and not

normal lymph node tissue, sparing a large number of lymphocytes for therapeutic use and at the same time enhancing the sensitivity of metastasis detection. In this thesis, this analysis strategy is extended to the micro-metastasis detection in lymph nodes draining penile cancer. We find that, using pan-cytokeratin staining of tumor cells the single cell preparation from lymph nodes of patients, we are able to identify metastasis in all cases also confirmed by standard pathological examination of other parts of the lymph nodes, and additionally we identify metastasis in two patients where no metastasis was found by standard measures. We also present preliminary data from an analysis of the cellular composition of the patient lymph nodes, and data on the possible reactivity of the lymph node T-cells to antigens found in autologous whole tumor lysates. As a large proportion of penile cancers are associated with HPV infection we also tested the reactivity of lymph node T-cells to the Gardasil® vaccine antigen preparation. The thesis also presents a clinical study extending earlier clinical immunotherapy studies on the expansion of tumor reactive, sentinel lymph node T-cells for the treatment of colorectal cancer. We performed a phase I/II clinical study on 71 colorectal cancer patients. No treatment-related toxicity was observed among the patients who received the T-cell therapy. The 9 patients with stage IV that received the sentinel node T-cell therapy showed an increased 24-month survival rate compared to the control group of 16 patients who received standard therapy and there was a tendency to increased overall survival after conclusion of the 33-month follow up. Our study demonstrates that tumor draining lymph node based T cell immunotherapy is feasible and safe for patients with colorectal cancer.

LIST OF SCIENTIFIC PAPERS

- I. Yunhuan Zhen, Xiaohui Liu, Yuan Yang, Bo Li, Jingling Tang, Qiangxing Zeng, Jiehu, Xingnan Zeng, **Lu Zhang**, Zejun Wang, Xiaoyun Li, Huixin Ge, Ola Winqvist, Pingsheng Hu, Jin Xiu.
Phase I/II study of adjuvant immunotherapy with sentinel lymph node T lymphocytes in patients with colorectal cancer. *Cancer Immunol Immunother* (2015) 64:1083 - 1093
- II. **Lu Zhang**, Jin Hu, A. Ali Zirakzadeh, Jesper Rosvall, Mats Hedlund, Ping Sheng Hu, Robert P.A. Wallin, Amir Sherif, Ola Winqvist
Detection of micro-metastases by flow cytometry in Lymph Nodes from patients with penile cancer. *BMC Urol.* 2018 Oct 5;18(1):86
- III. **Lu Zhang**, Jin Hu, A. Ali Zirakzadeh, Jesper Rosvall, Mats Hedlund, Ping Sheng Hu, Robert P.A. Wallin, Amir Sherif, Ola Winqvist
Investigations on immune responses against autologous tumor and human papilloma virus in lymph nodes from patients with penile cancer.
[Manuscript]

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LIST OF ABBREVIATIONS

ACT	Adoptive cell transfusion
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APC	Antigen Presenting Cell
AST	Aspartate transaminase
BCG	Bacillus Calmette-Guerin
CD	Cluster of differentiation
CEA	Carcinoma embryonic antigen
CRC	Colorectal Cancer
CTLA	Cytotoxic T lymphocyte antigen
CTLs	Cytotoxic T lymphocytes
ECOG	Eastern Cooperative Oncology Group
ELISPOT	Enzyme-linked immunospot
EpCAM	Epithelial cell adhesion molecule
FACS	Flow associated cell sorting
FASCIA	Flow cytometric assay of specific cell mediated immune response in activated whole blood
HPV	Human papilloma virus
IFN	Interferon
IL	Interleukin
LN	Lymph node
MHC	Major histocompatibility complex
NK	Natural killer
OS	Overall survival
PBL	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1

TACE	Transcatheter arterial chemoembolization
TCR	T cell receptor
Th	T helper
TIL	Tumor infiltrating lymphocyte
UBC	Urinary bladder cancer

1 INTRODUCTION

1.1 CANCER

Cancer is a leading cause of death across the world. It is estimated by the International Agency for Research on Cancer (GLOBOCAN) that approximately 14.1 million new patients were diagnosed with cancer and 8.2 million patients died of the disease in 2012 worldwide (1). When comparing the incidence rates of different solid cancers between more and less developed countries, they found that prostate, colorectal, female breast, and lung cancer frequencies are much higher in more developed countries while liver, stomach, and cervical cancers accounts for higher prevalence in less developed countries.

1.1.1 Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide, and each year more than 1 million patients are diagnosed with CRC (2). There are no gender differences in CRC and the overall 5-year survival is approximately 50%. Although in a recent study in America, the incidence rates decreased 1.9% among men and 1.7% among women, colorectal cancer remained as one of the top three common cancer types (3). Risk factors for the disease are including lifestyle, aging and inherited genetic disorders (4, 5). The standard treatment for CRC is combined surgery, chemotherapy, radiation therapy, and targeted therapy.

In patients (stage I–III) who received surgery, more than 1/3 of the cases developed tumor recurrence even with distant metastases (6). It has also been suggested that the 5-year survival rate is poor (<12.5 %) for patients with metastatic CRC (mCRC) (7).

1.1.2 Penile cancer

Although penile cancer is rarely occurred in developed nations, the incidence of which varies from 0.3 to 1 per 100,000 each year, it is much common in some areas of developing countries. Human papilloma virus (HPV) infection is estimated to cause 40-50% of all penile cancer (8). Most patients are diagnosed with metastasis (9). Surgery, radiation therapy and chemotherapy are the major therapies used. In spite of these treatments, most regional recurrences occur within 2 years after resection (10). The risk of recurrence suggests the benefit of neoadjuvant chemotherapy regimens is limited. From an investigation of survival trends from the national data base, the survival rates remain unchanged from 1998 to 2009 (11). Thus, additional treatment modalities are needed.

1.2 TUMOR IMMUNOLOGY

1.2.1 Tumor immunosurveillance

In the early days of modern immunology at the beginning of the 20th century, Paul Ehrlich hypothesized the anti-toxins that he had studied could work as magic bullets in fighting human diseases including cancer. This inspired scientists to look for such antibodies against cancer cells. In the 1950ies a number of researchers found evidence for immune reactions against autologous cancer cells and Thomas and Burnet (12) claimed in their immune-surveillance hypothesis that protection against cancer was one of the important functions of the immune system.

1.2.2 Tumor antigens

A poor understanding of the nature of immune recognition and the basic functions of the immune system hampered the progress in the tumor immunology in the first 2 decades after it had been found that experimental animals could reject transplanted tumors. Antibodies was in these days better characterized and for decades researchers were looking for tumor antigen targets on the surface of tumor cells recognized by antibodies, however, these efforts had little success. In the 1960ies it was recognized that the cellular immune response was mediating the anti-tumor response and later and with increasing understanding of the different lymphocyte populations that the thymus dependent T-lymphocytes were important for anti-tumor immunity. The way that T-cells recognize their antigen was not elucidated until mid-1980ies. It was then realized that T-cells recognize their antigen as peptide fragments of proteins bound to the major histocompatibility molecules. Identifying such antigens from tumors proved to be a big challenge but theoretically it was speculated that such antigens could be generated by several processes divided into tumor-specific antigens (TSA) and tumor associated antigens (TAA). Abnormal proteins are produced by tumor cells, due to mutation of the concerned gene. Mutations in proto-oncogenes and in tumor suppressors may directly lead to the occurrence of a tumor. Such mutated abnormal proteins are called tumor-specific antigens (TSA). Tumor associated antigens TAA recognized by T cells with a targetable molecule structure are characterized into 5 groups (13):

- 1) Tissue differentiation antigens: Tumor cells usually express antigens which carried the characteristics from their origin tissue after transformation. These antigens could only be used as targets when the tissues are not crucial parts of the body. For example Carcinoembryonic antigen (CEA) can mount specific T cell response

towards colorectal cancer but in addition induce transient colitis at the same time (14).

- 2) Mutant protein antigens: After several mutations were found in melanoma genes, genome sequencing (especially exomic sequencing) became popular in order to find new epitopes, neo-antigens (13).
- 3) Viral antigens: Cancers which are induced by a virus infection will express viral proteins during tumor development. These antigens offer interesting targets since they are not present in normal tissue. It is thus possible to target oncoproteins encoded by the tumor associated virus such as proteins derived from Epstein–Barr virus (EBV) (15) and human papilloma virus (HPV) (16).
- 4) Antigens produced by epigenetic changes: Expression of non-mutated proteins activated in several cancer types which triggered by epigenetic changes of the gene are mainly from germ line cells. These proteins are called cancer germline antigens or cancer-testis antigens (13). The encoded genes are inactive in normal non-germline tissues.
- 5) Antigens on non-transformed tumor vasculature and stroma: Tumors contain not only transformed cells but additional cell types including stromal cells and leukocytes (13). A study found targeting these cells beneficial for tumor treatment (17).

Two main strategies were used to identify the first tumor antigens, molecular cloning of genes and mutations recognized by T-cellines and mass spectrometric identification of peptides eluted from MHC molecules of tumor cells recognized by T-cellines. The first tumor antigen was identified by the group of Thierry Boon and was from an unknown protein which gene was named Melanoma Associated GENE-1 (MAGE-1) (18). The

first MHC class II-restricted antigen, tyrosinase, was found in a T cell line derived from a patient with melanoma (19).

1.2.3 Immunoediting

Along the studies of our immune system reacting towards tumor cells, the concept of immunoediting was brought up as a better explanation of the interaction between tumor and immune cells. It comprises of three phases: 1) elimination, in which the immune system recognize the tumor cells and eliminate them as proposed in the tumor immunosurveillance hypothesis; 2) equilibrium, when the interaction between the tumor and the immune system enter into a dynamic equilibrium, resulting in a contained but non-growing tumor state and 3) escape, where the tumor is capable to resist and avoid immune detection and/or elimination leading to tumor growth (20).

1.2.3.1 The elimination phase

According to the tumor immunosurveillance hypotheses, it is possible for immune cells to eliminate developing tumor cells, a mechanism involving both the innate and the adaptive immune responses. It starts when innate immune cells such as macrophages, dendritic cells and NK cells are recruited towards the tumor site as a result of pro-inflammatory molecules and chemokines produced in the tumor microenvironment. As soon as the summoned cells reach the tumor site, molecules expressed by the tumors such as NKG2D will be recognized and then IFN- γ will be secreted (21). As a result of the IFN- γ secretion processes, the destruction of the tumor part could lead to further reaction by adaptive immune system. Recruited DCs can thus obtain tumor antigens by ingesting tumor cell debris and then migrate to the draining lymph nodes where they present the tumor antigen to CD4⁺ and CD8⁺ T cells (22, 23). It then leads to the next

step where tumor- specific T cells will home to the tumor site and eliminate the tumor cells (20).

1.2.3.2 The equilibrium phase

A few cancer cells variants may survive from the elimination phase and enter the equilibrium phase which is probably is the longest among the three phases. It could be better described as a Darwinian selection process between the tumor and the immune system. During this phase, tumor growth is restricted by immune-mediated cell death but no complete elimination occurs. T cells, IL-12 and IFN- γ have the major roles for maintaining tumor cells at steady state which results in a tumor dormancy phase (24).

1.2.3.3 The tumor immune escape

In this phase, tumor cells are capable to circumvent immune recognition and/or destruction resulting in tumor expansion and clinical appearance. This capacity is considered a part of the “hallmarks of cancer” criteria (25). It occurs through the involvement of cellular and molecular changes within the tumor cells which varies from patient to patient and between tumor types (26). These diversity conditions which leads to tumor immune escape require distinct immunotherapeutic strategies for maximal therapeutic effect (27). Some known and newly discovered tumor immune escape mechanisms will be discussed below:

Lack of immune recognition and destruction

Tumors which are absent with MHC expression or deficient in antigen presentation might be able to escape elimination by tumor-specific T cells. MHC class I molecules has been noticed to be downregulated in approximately 20-60% of solid tumors (28). In virus-associated cancer cases, i.e. HPV is capable of downregulating MHC (HLA) class I and therefore reduce recognition by CD8⁺ T cells (29).

Although tumors can express mutated and non-mutated antigens which can potentially elicit immune responses, they can escape from immune surveillance by losing their antigenicity. Loss of antigenicity may arise since cancer cells which lack or mutate immunogenic tumor antigens benefit from escaping immune recognition (30).

Furthermore, an abnormal IFN- γ receptor signaling pathway in tumor cells will allow them to be insensitive towards IFN- γ (31). Also, the expression of STAT3, which induces anti-apoptotic signals in tumor cells and mediates immunosuppression, provides another way to avoid immune destruction (32). Besides, in the tumor microenvironment, lactate is produced and could selectively inhibit FAK family-interacting protein of 200 kDa (FIP200). Lacking of FIP200 leads to apoptosis of the naïve T cells which will restrain immune responses towards tumor (33). Recently, tumor cells were found to have the capability to restrict TNF cytokine signaling in CD8⁺ T cells resulting in tumor immune evasion(34).

Induction by immune suppression

To induce immune suppression, the tumor itself could synthesize several suppressive molecules including: Indoleamine 2,3-dioxygenase (IDO), an enzyme which suppress T cell proliferation by mediating the degradation of the essential amino acid tryptophan (35); Suppressive cytokines like TGF- β which could suppresses the transcription of perforin and granzymes (36); Suppressive chemokines such as CXCL1, which recently demonstrated its role in suppression of T cell infiltration and decreased sensitivity to immunotherapy (37).

Another way to induce immune suppression by the tumor is the hijacking of immune checkpoints. For example, Programmed cell Death protein-1 (PD-1) could by binding to its ligands, PD-L1 or PD-L2, leading to decreased phosphorylation of TCR signaling

molecules and limited T cell effector function (38). Immune checkpoints will be discussed further below.

Moreover, in the tumor microenvironment, regulatory T cells which are essential to remain immunological self-tolerance and abundant in tumors (39), secreting IL-10, TGF- β and expressing the checkpoint molecule CTLA-4 thereby inducing immune suppression.

1.3 IMMUNOTHERAPY OF CANCER

The treatment of cancer has undergone evolutionary changes along with further understanding of the tumor biological process. Surgery, Radiation therapy and Chemotherapy are the major strategies to treat cancer. In most cases of solid tumor, surgery is the primary therapy and may be sufficient in local disease. However, the prognosis is poor when the metastasis of tumors takes place. Even combination of radio- and/or chemotherapy, the likelihood of a cure is still less than 50% for many solid tumors (2). Apparently, it is necessary to explore new methodologies in treating cancer patients. Immunotherapy is a new strategy that may contribute to this.

Nowadays, immunotherapies fall into three main groups: cellular, antibody and cytokine mediated therapies. Using the immune system to treat cancer could be traced back to 1891, when the surgeon William B. Coley accomplished the first systematic study in this field, long before the formulation of the tumor immune surveillance theory. He observed the spontaneous tumor regressions in patients who developed bacterial infections. Then he succeeded to cure more than 10% of patients with inoperable sarcomas by bacterial vaccine (40). A descendant of Coley's toxin is the Bacillus-

Calmette- Guérin (BCG), an attenuated strain of mycobacteria, which is used for treating superficial urinary bladder cancer (UBC) (41). Depending on the concurrent presence of CD4⁺ and CD8⁺ T cells, the efficacy of this treatment could be different (42). These approaches first activate the innate immune system unspecifically, and then rely on the activation of the adaptive immune system initiating a tumor specific immune response. Antibody therapy was first described as the magic bullet by Paul Ehrlich, using antibodies specific to a disease. In 1975 pure monoclonal antibodies were capable to produce as a therapy and the hybridoma technology was introduced (43). Remarkably, Rituximab, the first antibody drug was approved by the FDA to treat follicular lymphoma in 1997. Till now, 11 additional monoclonal antibodies have been approved for cancer therapies. Another non-specific methodology is the administration of cytokines. For instance, the usage of high dose IL-2 can lead to durable remission in some melanoma patients (44), a mechanism likely mediated by NK cells.

1.3.1 Adoptive T cell therapy

The concept of adoptive T-cell therapy was brought up by Rosenberg and colleagues, who were inspired by the regressions of tumors with high-dose IL-2 administration to melanoma patients. Lymphokine activated killer (LAK) cells were described in 1980, and they are white blood cells that stimulated with IL-2 to kill tumor cells. This cell subtype was different from the natural killer cell population in their functions: LAK cells have the capacity to eliminate both allogeneic and autologous cancer cells, while friendly to normal cells (45). In clinical trials, 31% melanoma patients who had received LAK plus IL-2 demonstrated immune responses (46). Later, from animal studies, tumor infiltrating lymphocytes (TIL) were found to be 50-100 times powerful

than LAK cells in treating metastatic disease, and successfully reached a large expansion *ex-vivo* (47). Followed by a pilot study in humans, immune responses were found in melanoma patients who received TIL plus IL-2 treatment. Then propagation of tumor TIL from solid tumor biopsies became possible, and a new methodology of treatment appeared (48, 49). Furthermore, a study demonstrated a treatment which combined transferring autologous T cells with high-dose IL-2 therapy on melanoma patients who previously received nonmyeloablative lymphodepleting chemotherapy. In this study, antigen specific T cells were found growing rapidly *in vivo* and to be able to invoke immune responses towards both tumor cells and normal tissue expressing the same antigen (50). The results demonstrated the effectivity of TIL therapy and indicated the potential target which could be used in immune therapy on other tumor types.

Several attempts were made on other solid cancers using adoptive T-cell therapy, but the studies are small and with less promising results than in melanoma. For example, a study on colorectal cancer found no differences in prognoses after receiving TIL treatment (51).

Recently, combining virus with onco-protein reactivity, HPV- Targeted Tumor-Infiltrating T cells were demonstrated in cervical cancer patients and 3 of 9 patients developed complete responses (52).

1.3.2 Draining lymph node based immunotherapy

Tumor draining lymph nodes (LNs) were first identified by Cabanas in penile carcinoma (53). The histopathological status of the draining LNs may represent the entire lymphatic field. They can be identified by injected a tracer (either a radioactive compound or a blue dye) to the tumor. This concept is widely used for staging of breast

cancer and melanoma, and may influence the extent of surgery and postoperative treatment planning. Draining lymph node detection has been introduced in CRC and UBC suggesting great potential for staging (54-56). Furthermore, immune responses to autologous tumor cells were found in LNs of colon and UBC patients (55, 57) demonstrating the recognition of tumor antigens by T cells in LNs. This suggests that the tumor draining LNs may be a useful source for harvesting and expanding T cells as new approach to treat CRC and UBC.

Single cell suspensions from LNs were cultured with autologous tumor extract combined with IL-2 *in vitro*. Tumor specific T cells were expanded and then transferred back to the patients. Approximately half (4/9) of stage IV CRC patients, who received LN based immunotherapy, responded with complete tumor regression (58). Therefore further investigation of this therapy is warranted.

1.3.3 TCR Transgenic therapy

T cell can be manipulated and redirected to tumor antigen by genetic engineering TCRs (TCR therapies), which include endogenous TCR and genetically modified TCR which can enhance antigen specificity (59) (Figure 1.).

Expression of a cloned TCR with targeted affinity for tumor antigen was used in the earliest clinical trial using engineered T cells. A TCR could recognize either intra- or extra- cellular antigens by MHC presentation. However, many tumors downregulate MHC class I expression for immune evasion, thus limiting the efficacy of TCR-engineered T cells. Several attempts have been made to use artificial receptors, such as chimeric antigen receptors (CARs) in order to increase the specificity of engineered T cell (60).

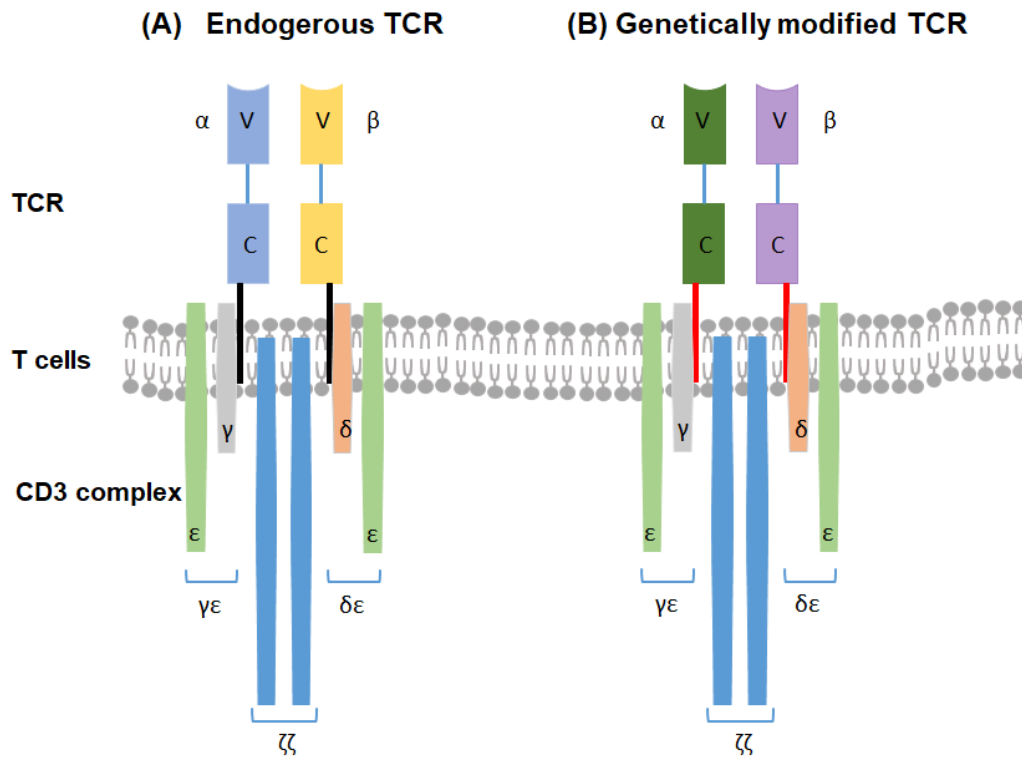


Figure 1. Genetically modified TCRs for cancer immunotherapy.

Figure is adapted from (59). (A) Endogenous TCR: Genetic engineered of the endogenous TCR could manipulate T-cells with higher specificity and affinity towards tumor antigens. (B) Genetically modified TCR: T cell encode new TCR α and β chains after transferred specific gene sequences. Also, there could be transmembrane changes (red bars). Modifications including adding a disulphide bridge (ss) were made to minimize mispairings of the interchain of the endogenous TCR.

Studies of targeting B cell malignancies demonstrated good responses to treat patients with relapsed and refractory B cell acute lymphoblastic leukemia (B-ALL). The most common B cell target is the extracellular glycoprotein CD19. Several groups reported more than 80% of patients with this disease to respond to CART19 cells (60).

Furthermore, the overall response rates were 50-80% in clinical trials (61, 62). Besides, CD20 and CD22 which are commonly expressed in B-ALL and non-Hodgkin lymphoma are also considered as targets. Multiple clinical trials are under way (60).

In the process of B cell development, cell can express either immunoglobulin- κ (Ig κ) or Ig λ light chains. It has a normal range of the ratio between Ig κ^+ and Ig λ^+ cells in human. When an abnormal ratio occurs, it indicates a clonal expansion of Ig light chain-restricted population. This could be a target for engineered T cell therapy and is presently tested in early phase clinical trial (60). Besides, NY-ESO-1 as one of the cancer-testis antigens which can be highly immunogenic is upregulated on plasma cells myeloma (63). When treating patients with advanced multiple myeloma by NY-ESO-1-specific TCR-engineered T cells, 80% of patients (16/20) responded and a median progression-free survival of 19.1 months was observed (64).

The success of engineered T cell treatment for B cell malignancies indicated the potential value of this approach for other types of hematological malignancies and solid tumors. For example, B cell maturation antigen (BCMA) is expressed in most incidents of multiple myeloma and absence in non-plasma cells is considered as a target antigen. There are two phase I trials ongoing to investigate the safety, feasibility and efficacy of BCMA-CAR T cells target to multiple myeloma (60).

With regard to the treatment towards solid tumor, off-tumor effects are less tolerable than in hematological malignancies. Moreover, most potential solid tumor targets are non-specific and could be found on healthy tissue. It is therefore challenging to find a proper target in solid tumor. To date, some overexpressed antigens in tumor tissue were considered as targets of engineered T cell therapy, such as interleukin 13 receptor

$\alpha 2$ subunit (IL13R $\alpha 2$), mesothelin, GD2 and human epidermal growth factor receptor 2 (HER2) (60).

CEA-specific TCR-engineered T cells were setup (65) and used in the treatment of 3 CRC patients. Decreasing serum CEA levels (74–99%) was observed in all patients and one patient experienced an objective regression of cancer metastasis in the lung and liver. However, a dose related toxicity was induced and represented as severe transient inflammatory colitis in all patients who received the therapy (14).

For virus associated cancer, virus antigen might be a better target. To date, a phase I/II clinical trial conducted E6-specific engineered T-cell therapy on 12 patients with HPV-associated cancers (including 6 cervical, 4 anal, 1 oropharyngeal, and 1 vaginal). The safety dose was found up to 2×10^{11} cells. Two patients with anal cancer who received the highest cell dose had partial tumor responses after treatment and remained 6 and 3 months. The patient who received 6-month response experienced complete regression of a tumor (66). The results indicate the potential value of E6-specific engineered T-cell therapy in treatment of HPV-associated cancer.

1.3.4 Check point inhibitors CTLA-4 and PD-1

Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) is a cell surface molecule which is expressed on activated CD4 and CD8 T cells and it has a higher affinity than CD28 therefore competing with binding to the B7 molecules, leading to the loss of co-stimulation thus negatively regulate T cell activation(67). Programmed cell death-1 (PD-1), expressed on the surface of activated T cells, can interact with its ligands: programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2), will

lead to T cell inactivation and provide a possible mechanism for tumor induced immune escape (68). Drugs blocking these molecules have been developed and they are called immune checkpoint blockers (ICBs). ICBs have demonstrated their effectivity in clinical trials including metastatic melanoma, renal-cell carcinoma (RCC), classic Hodgkin's lymphoma (HL), advanced non-small-cell lung cancer (NSCLC), UBC, head and neck cancer, et cetera (69).

1.4 IMMUNOTHERAPY IN COLORECTAL CANCER

1.4.1 Microsatellite instability (MSI) in CRC immunotherapy

In an early study conducted using CTLA-4 antagonist mAb (Tremelimumab) in CRC patients responses were demonstrated. It suggested the possibility to use immune checkpoint inhibitors in treatments of CRC (70). However, the checkpoint inhibitor is not as effective as in other tumor types (71). Recent studies sub grouped the CRC for which presenting dysfunctional mismatch repair proteins within a tumor as microsatellite instability (MSI) (72). MSI accounts for approximately 15% of all CRCs and the prevalence of which is dependent on the tumor stage. Interestingly, an upregulation of check point inhibitory molecules was found in MSI-H CRC patients (73). In November 2015, FDA granted pembrolizumab Breakthrough Therapy Designation for MSI-H CRC. From 2017, NCCN guidelines have been modified to add both nivolumab and pembrolizumab as choices to treat metastatic and unresectable MSI-H CRC.

1.4.2 Therapeutic vaccines

1.4.2.1 *Autologous vaccines*

Autologous vaccines use the autologous tumor cells from the patients which contain all relevant tumor-associated antigens (TAAs) for therapy. Since whole tumor cell vaccines has limited efficacy in the clinic, there are two modified vaccines which are combined with BCG vaccine (74) and Newcastle disease virus (NDV) (75) that now are in clinical trials with the idea to provide adjuvant activation to enhance TAA responses.

1.4.2.2 *Peptide vaccines*

Peptide vaccines are normally 8-10 amino acids long, which could elicit specific T cell responses against Tumor antigen and be co-administered with adjuvants thus augmenting the tumor specific immune response. The common targets for peptide vaccines in CRC treatments are CEA, epidermal growth factor receptor (EGFR), mucin 1, squamous cell carcinoma antigen recognized by T cells 3 (SART3) and Survivin-2B (73). However, due to several limitations such as weak immunogenicity, HLA haplotype restriction and immune-evasion, most clinical trials have failed (76). There are some trials ongoing, using peptide vaccines target to multiple epitopes with longer amino acid sequences trying to deal with the limitations (77).

1.4.2.3 *DC vaccines*

Dendritic cells (DC) as an important antigen presenting cells can present multiple TAAs by MHC class I and II molecules. They are involved in programming and regulating the immune response against the tumor by providing co-stimulatory signals and cytokine secretion. DC based vaccine has been studied for decades for the treatment of cancer. Recent attempts have tried loading DCs harvested from patients with TAAs, lysis of

tumor cells, apoptotic tumor cells, tumor RNA or whole tumor cells *in vitro* followed by transfusion back to patients once cells are activated (78).

CEA as a TAA was found in most of the CRCs. There are multiple early phase trails demonstrating that CEA loaded DCs are safe and effective in generating a CEA specific tumor response. However, a large phase III trial is warranted to support the clinical benefit of DCs vaccines (73).

1.4.2.4 Viral vector vaccines

The usage of viral antigen vaccines can induce a robust tumor specific and substantial immune response. Since viral vector vaccines have high transfection efficiency including recombinant lentiviruses, poxviruses, adenoviruses and retroviruses, they may be better than peptide vaccines in generating tumor responses (73).

1.4.3 Additional Immunotherapy Combinations

Several early phase clinical trials are ongoing with combined block of suppressive immune factors, for instance LAG-3 or indoleamine 2,3-dioxygenase(IDO) with PD-1 or PD-L1 inhibitors. In addition, reagents which have the capacity to stimulate immune reactivity such as KIR and 4-1BB (CD137) are presently studied in several combinations in clinical trials (71).

1.5 HUMAN PAPILLOMA VIRUS RELATED CANCER

Human papillomavirus (HPV) is a DNA virus belonging to the papillomavirus family that has the capability to infect humans. HPV infection accounts for 5.2 % of all cancers

such as cervical cancer, oropharyngeal cancer and penile cancer, worldwide (79). More than 170 HPV types have been identified (80). Varied from the risks of inducing malignancy, HPV types are grouped into high or low-risk types. The high-risk group for instance HPV-8, -16, -18 and -31 could cause cancer (81), whereas the low-risk types such as HPV-6, -11 can lead to benign warts and lesions without transformation. However, a case report with invasive verrucous carcinoma of the penile reported association with the low risk HPV 11 (82). HPV infection is well known to cause cervical, vulvar, head and neck cancer. However, HPV associated penile cancer is seldom studied.

Studies found HPV DNA in both primary tumors and metastases suggesting HPV might be involved in tumor progression (53) (79).

The HPV genome encodes two shell proteins (L1 and L2) and no less than six early proteins (E1, E2, E4–E7), which support viral DNA to replicate and newly produced virus particles to be assembled in the infected cells (83). HPV16 genome structure was displayed in Figure 2.

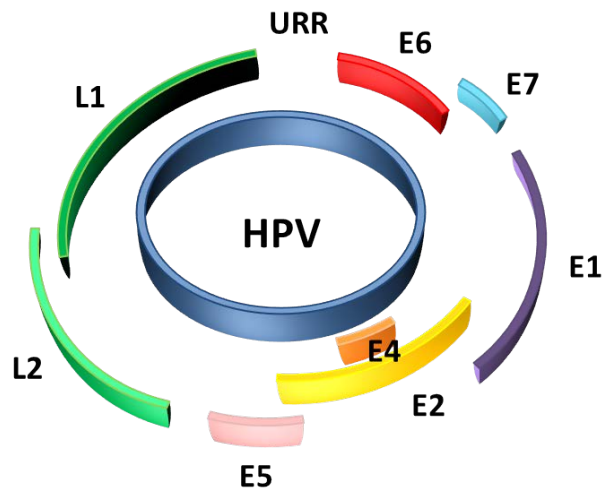


Figure 2. HPV genome organization.

The genome structure of a typical high-risk HPV was displayed. Genes were grouped according to the early or late phase of expression during the viral life cycle. Adapted from Ref. (84)

Early genes: encoding six nonstructural proteins which are involved in viral replication and transcription.	
E1: Replication	E5: Immune evasion
E2: Replication and transcription	E6: Binds p53
E4: Viral release	E7: Binds pRB
Late genes: encode viral structural proteins.	
L1: Major capsid protein	L2: Minor capsid protein
URR: upstream regulatory region (URR) contains promoter, enhancer elements and the viral origin of replication (ORI).	

Animal studies suggest HPV E6 and E7 onco-proteins could affect cell growth control by inactivating the tumor suppressor gene products p53 and Rb, respectively (85). Thus E6 and E7 are commonly studied in HPV associated cancer immunotherapy (52, 86, 87). A study compared the efficiency of different HPV antigen specific T cells in eliminating autologous HPV⁺ tumor cells from patients with cervical cancer. The study concludes that L1-specific CD8⁺ cytotoxic T lymphocytes (CTLs) are the same effective as E7-specific CD8⁺ CTLs (88). HPV vaccine studies have demonstrated that Gardasil could be highly efficacious in protecting women against HPV 16/18, which can cause high grade cervical intra epithelial disease (CIN2/3) (89). These findings suggest that Gardasil is of great value for immunotherapy.

1.5.1 Potential target for immunotherapy

Studies on penile cancer have demonstrated that the most prevalent strain is HPV16 (90). The HPV virus has developed means to avoid T cell recognition by devoting genes to intervene with HLA class expression. For instance, the early HPV proteins from E5 and E7 can reduce the expression of HLA class I (91, 92). Furthermore, one study on head and neck cancer suggests that E2 might be involved in the downregulation of HLA class I (93). Therefore, it would be highly interesting to investigate these proteins and their expression in patients with penile cancer, since they may be potential antigen targets for immunotherapy of penile carcinoma.

1.6 CYTOKINES IN T CELL EXPANSION

Cytokines are major regulators of innate and adaptive immunity that allow cells to

interact. There is a cytokine family belonging to type I cytokines, which share the signaling by the receptor γ chain (also known as IL-2R γ and CD132) (94) The cytokines include interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21 named as the common cytokine receptor γ -chain (γ_c) family. Interleukin-2 (IL-2) was the first discovered cytokine in this family. After the success in treatments of metastatic melanoma and renal cancer with high or low dose of IL-2 infusion, application of cytokines from the IL-2 family especially interleukin (IL)-2, IL-7, IL-15, and IL-21 became one important cancer immunotherapy research area (95). Here, I will focus on IL-2, IL-7, IL-15 and IL-21(Figure 3) for further discussion.

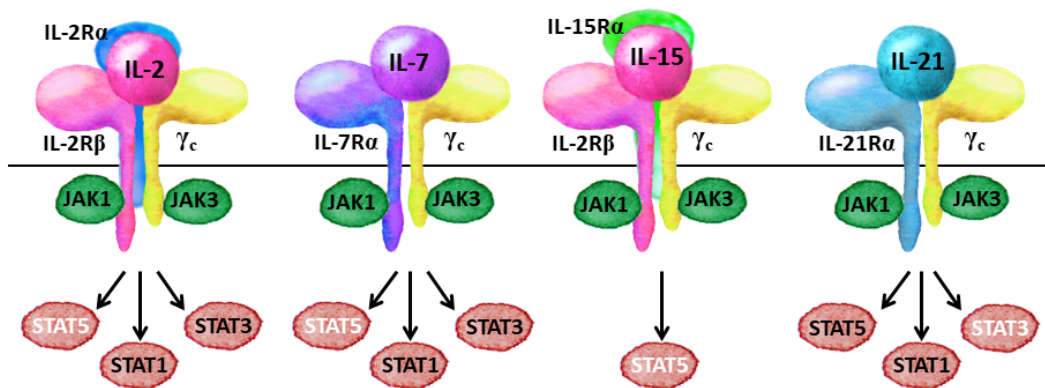
Cytokines produced by:

IL-2: T cells and DCs

IL-7: stromal cells, epithelia cells and mast cells

IL-15: monocytes, DCs and epithelia cells

IL-21: CD4⁺ T cells and NKT cells



Receptor expressed by:

T cells, B cells and NK cells

T cells, pre-B cells and DCs

T cells and NK cells

T cells, B cells, NK cells and DCs

Adapted from Rochman Y, et al. Nat Rev Immunol. 2009

Figure 3. Receptors for γ_c family cytokines.

Among these cytokines, only IL-2 and IL-15 have three receptor chains. They also share the common cytokine receptor γ -chain (γc ; also known as IL-2R γ) and IL-2R β . The receptors activate Janus kinase 1 (JAK1) and JAK3. The main downstream activating signal transducer and activator of transcription (STAT) protein for each cytokine is shown in white.

1.6.1 IL-2

IL-2 is mainly produced by CD4⁺ T cells during normal condition, and it promotes CD4⁺ and CD8⁺ T cell proliferation (96) thus firstly described as “T cell growth factor”. Later, its capacity of inducing differentiation of activated B cells, and promoting the cytotoxic activity of natural killer (NK) cells were found (97). IL-2 also induce Fas-mediated apoptosis in CD4⁺ T cells (IL-2 induced cell death) in a way eliminate T cells which are over stimulated thus avoid to cause autoimmunity (98). Additionally, IL-2 R α as one of the IL-2 receptor subunits, is highly expressed in CD4⁺CD25⁺FoxP3⁺ regulatory T (Treg) cells which could suppress activities of the T helper cells allowing self- tolerance to be maintained (99).

1.6.2 IL-7

IL-7 is essential by providing a homeostatic support for T cells to grow and survive (100). IL-7 could promote naïve T cells survival by 1) activating the pro-survival phosphoinositide 3-kinase (PI3K)-AKT signaling pathway resulting in the inhibition of apoptosis, by upregulating the anti-apoptotic proteins, myeloid cell leukemia 1 (Mcl-1) and B-cell lymphoma 2 (Bcl-2) (101) ; 2) maintaining the T cells at trophic state and their glucose metabolism (102).

1.6.3 IL-15

IL-15 is another major homeostatic cytokine, which particularly regulate the IL-2R β^{hi} LY49 $^+$ subset of memory CD8 $^+$ T cells (103). IL-15 has similar structure to IL-2: both have IL-2/15R β and γ_C thereby implicating similar effects (104). However, IL-15 has its unique characteristics. Unlike IL-2 which is expressed in T cells and can regulate T cell activation, IL-15 emerges when cells express IL-15R α such as activated monocytes. It has an important role for memory and naive CD8 $^+$ T cell and NK cell to survive whereas no effect is seen on Treg cells (97).

1.6.4 IL-21

IL-21, which is mainly produced by CD4 $^+$ T cells, stimulates B cells, memory T cells, and bone marrow progenitors. It can promote cytotoxicity of CD8 $^+$ T cells and NK cells while be capable to suppress Tregs (105, 106). Later, IL-21 was found to augment the frequency of antigen-specific T cell compared with other cultures adding IL-2, IL-7 and IL-15 separately during expansion. IL-21 was found to mainly influence naive T cells and a population of CD45RO $^+$ CD28 $^{\text{high}}$ CD8 $^+$ T cells was largely expanded (107). One study found that IL-21 can synergize with IL-7 to augment cytotoxic T cells expansion and anti-tumor function (108). Studies on naïve T cell also suggests with the help of IL-21, IL-7/15, they could induce antigen-specific activation and further expand the cells (109).

2 AIMS OF THE THESIS

The overall aim of this thesis was to study lymphocytes from tumor draining lymph nodes and their reactivity towards tumor and virus antigens, in order to better understand antigen specific T cells responses and find potential ways to expand them for future immunotherapy.

The specific aims of each paper were:

Paper I. To investigate the safety and feasibility of tumor draining lymph node based T cell therapy in the patients with colorectal cancer.

Paper II. To examine if staining of pan-cytokeratin AE1/AE3 positive cells through flow cytometry is a sensitive and stable way to detect metastasis in the lymph nodes from patients with penile cancer to prepare for sentinel node based immunotherapy.

Paper III. To investigate the characteristics of lymphocytes from tumor draining lymph nodes from patients with penile cancer and the immune response towards tumor and HPV antigens.

3 MATERIALS AND METHODS

3.1 PATIENTS

3.1.1 Patient characteristics

Paper I is a phase I/II study of immunotherapy on patients with colorectal cancer. 71 CRC patients who underwent radical surgery (stage I–III, n = 46) or palliative surgery (stage IV with non-resectable synchronous metastases, n = 25) were recruited. 55 patients (Stage I-IV) received immunotherapy, including 37 males and 18 females, aged 32-74 years. The Phase II study followed stage IV patients by routine follow up programs including patients who received immunotherapy (n=9) and controls (n=16).

Paper II and Paper III both encompass penile cancer patients. Paper II is focused on staging of the lymph node in patients with penile cancer using flow cytometry. 5 patients, aged 70-80 who underwent surgery at Södersjukhuset, Stockholm and Norrlands Universitetssjukhus, Umeå, Sweden, (2014-2015) were included in this study. Paper III is a study on lymph node derived lymphocytes from patients with penile cancer and their reactivity to tumor antigen and human papillomavirus (HPV) antigen. 11 patients, 50-84 years old, from Södersjukhuset, Stockholm and Norrlands Universitetssjukhus, Umeå, Sweden, (2013-2015), were included in this study.

3.1.2 Patient follow-up

Patients with colorectal cancer described in paper I, who classified as stage IV were followed every 3 months in the first year and from second year were followed every 6 months. The disease status was assessed through physical examination, the serum level

of CEA, chest CT, abdominal CT and colonoscopy. In paper II and paper III are designed mainly for comparison current status combined with relatively short follow-up time (the latest follow-up in 6 August 2016), however in paper III we could still find the potential clue in explaining the outcome of the patients.

3.1.3 Tumor draining lymph node detection and specimen preparation

In study I, tumor draining lymph nodes were detected by injection of patent blue dye, and in study II and III, a radioactive tracer was added and combined with patent blue injection. In paper I-III, lymphocytes from peripheral blood were isolated by ficoll-hypaque (Pharmacia, Amersham). Single cell suspensions from tumor draining lymph nodes were acquired by gentle pressure using a loose-fit glass-homogenizer. Cells were washed twice and resuspended in either AIM V® (Life technologies) for culturing or PBS containing 2% FCS and 0.05% NaN₃ (FACS buffer) for flow cytometry analysis. Tumor cells were obtained from tumor tissue using GentleMACS Dissociator (Miltenyi Biotec) in 10 ml RPMI 1640 medium (Sigma), containing 1% collagenase/Hyaluronidase solution (StemCell Technologies). Some tumor tissue was apart and saved in RNA later (Ambion, Austin, TX, USA) for HPV DNA detection by PCR.

3.2 CELL CULTURE AND PREPARATION

3.2.1 Cell culture for immune investigation

In paper I, Single-cell suspensions obtained from tumor draining lymph nodes were resuspended in X-VIVO™ 15 serum-free cell culture medium (LONZA) at a density of 4×10^6 cells/ml with 1000 IU/ml recombinant human Interleukin-2 (Shuanglu, China). Cells were stimulated twice by adding autologous tumor lysate as described previously (58).

In paper III, PBMC, lymphocytes from draining lymph nodes and TILs were tested at 0.5 million cells/tube using tumor homogenate, Gardasil® (0.1-1.0 µg/ml) or Pokeweed mitogen (5 mg/ml) (Sigma) in medium for 7 days before later analysis.

3.2.2 Cell line culture and preparation for cell spiking in mixed culture

In paper II, the HeLa cell line CCL2 (HPV18 positive) was cultured in RPMI 1640 media (Sigma, Stockholm, Sweden), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Hyclone, Uppsala, Sweden), and 1% L-glutamine (Hyclone). The cells were detached by Trypsin-EDTA solution (Sigma). Harvested HeLa cells were added to PBMC and diluted in steps of three (3%, 1%, 0.33%, 0.11%, respectively), kept in FACS buffer. PBMCs alone were used as negative control.

3.2.3 Adoptive transfusion

Cultured lymphocytes were harvested, washed twice with saline solution then transferred to a sterile plastic bag containing 200 ml of saline solution and 1 % human

serum albumin (CSL Behring GmbH, Germany). The cells were intravenously transfused based on the blood transfusion guidelines of the hospital. The overall workflow of this clinical trial is depicted in Figure 4.

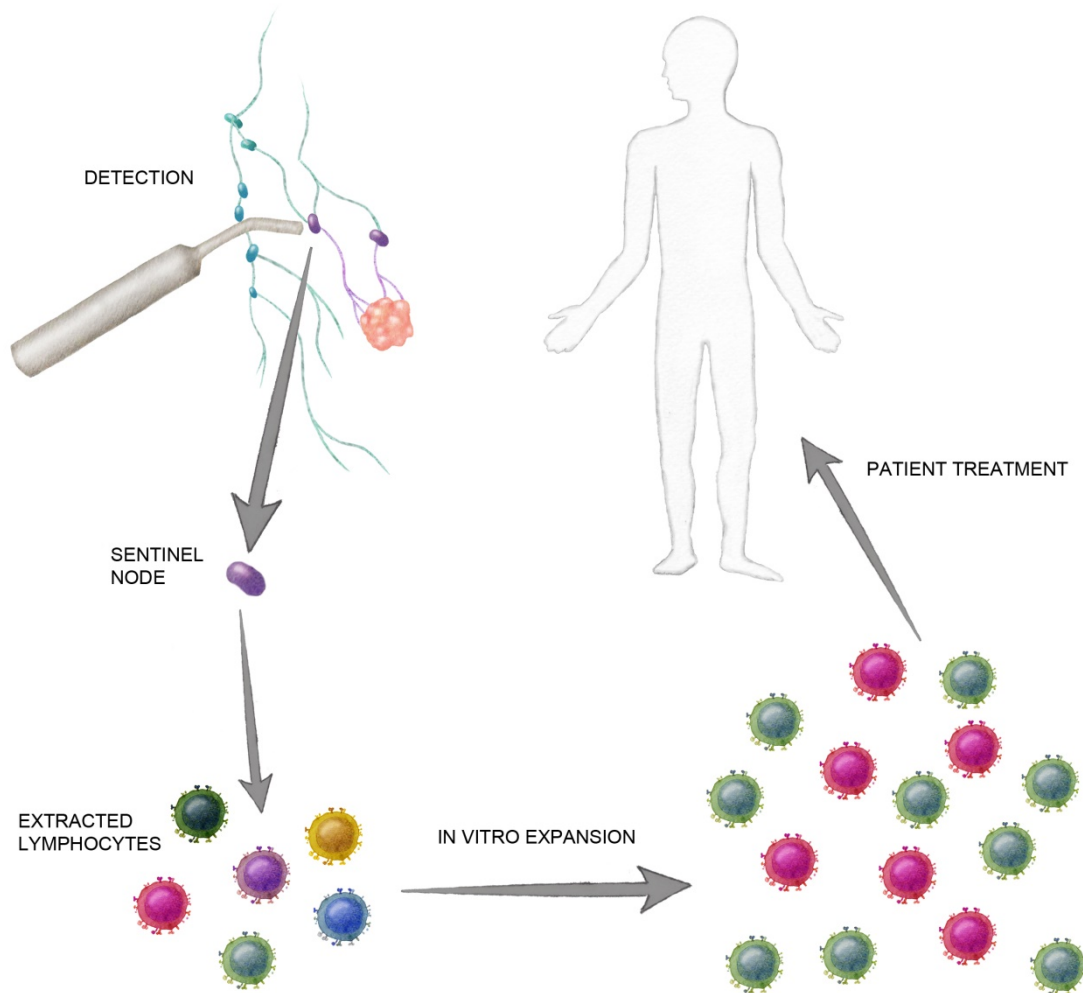


Figure 4. Overall workflow of the T cell expansion, paper I.

Tumor draining lymph nodes (sentinel nodes) were detected and harvested during the operation. Lymphocytes which extracted from the sentinel nodes were stimulated twice with the autologous tumor lysates and further expand in vitro together with IL-2. In the end of the expansion, enlarged tumor specific T cells were transferred back to the patient.

3.3 IMMUNOLOGICAL EVALUATION

3.3.1 Flow cytometry

For surface staining, single cell suspension obtained from peripheral blood, lymph nodes (paper I-III) and tumor tissue (paper I) were labelled with antibodies as indicated in the respective materials and methods part of paper I-III. In paper III, activation was measured by blast transformation (day 7) and cell surface markers according to FASCIA (110). Intracellular staining was performed using cytofix/cytoperm buffer (Becton Dickinson) for permeabilization as the beginning, then washed with 0.3% saponin (Sigma) in FACS-buffer followed by incubation with direct conjugated anti-human pancytokeratin AE1/AE3 antibody(eBioscience), or primary antibodies (E-cadherin antibody and anti-CK5/CK6 (Dako)). After incubation, cell suspensions were washed in saponin added FACS-buffer. For indirect conjugated antibodies, a secondary antibody (goat-anti-mouse which conjugates with allphycocyanine (Jackson Immunoresearch)) was stained. Acquisition of flow cytometry data was performed on an FC500 flow cytometer (Beckman Coulter) (Paper I) and an LSR-FORTESSA (Becton Dickinson) (Paper II and Paper III). Data was analyzed by CXP software (Paper I) and FACS DIVA software (Paper II and Paper III).

3.3.2 ELISPOT assay (Paper I)

To evaluate the antigen specific T cells by detect the interferon gamma (IFN- γ) releasing, using an enzyme-linked immunospot (ELISPOT) assay kit (Mabtech AB, Sweden) according to the manufacturer's protocol. The data was automated evaluated by AID FluoroSpot Reader System (Autoimmun Diagnostika GmbH, Germany).

3.4 POLYMERASE CHAIN REACTION (PCR) (PAPER III)

DNAs were extracted using DNAeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. Primers specified in paper III were used for detection of the L1-region from HPV16 and HPV18. HPV18 DNAs were obtained from HeLa cell line and HPV16 transformed cell DNA (Advanced Biotechnologies) were used as positive control. The assay was performed by Bio-Rad T100 following the standard PCR protocol.

3.5 SAFETY MEASUREMENTS OF TRANSFUSION

As a Phase I/II study describe in paper I, it is important to ensure no tumor cells were transferred back to the patients. Therefore, an EpCam staining was conducted using flow cytometry to exclude the presence of tumor cells (111).

In order to check that there was no contamination in any of the cell cultures, 5 ml of culture medium was removed for a bacterial and fungal contamination test using BACTEC 9120(Becton–Dickinson) and the endotoxin levels were measured with the Limulus reaction, these assays were taken 2 times on one week before transfusion and on the day of transfusion separately.

Transfusion-related toxicity was evaluated post-cell transfusion by Common Terminology Criteria for Adverse Events (CTCAE) 3.0 criteria.

3.6 STATISTICAL ANALYSIS

In paper I, surface markers changes and IFN- γ release between the groups were assessed by Student's t test or one-way ANOVA. Comparison of Categorical variables

were measured by Chi-squared and Fisher's exact tests. Influence of adoptive T cell therapy on OS was dominated with Kaplan–Meier curves. Log-rank test was used for evaluating the significance of the difference between two groups. In order to evaluate the sensitivity of flow cytometry in metastasis detection, comparisons between added and detected cells were assessed using linear regression (paper II). To investigate the links between the phenotypes of the cells obtained from tumor draining lymph nodes, correlations were measured by spearman r. The significance of the difference of CD4/CD8 ratio between HPV positive and negative patients was demonstrated by two-tailed t test (paper III). Statistical calculations were done in GraphPad Prism (Version 5.04). P-values <0.05 were considered as significant.

4 RESULTS AND DISCUSSION

4.1 TUMOR DRAINING LYMPH NODE DERIVED LYMPHOCYTE IMMUNOTHERAPY IS SAFE AS AN ADJUVANT THERAPY IN TREATMENTS OF COLORECTAL CANCER (PAPER I)

Previously we have demonstrated that lymphocytes from tumor draining lymph nodes have stronger immune responses towards autologous tumor lysate compared with TIL and lymphocytes from peripheral blood (57). In this study we aimed to conduct a Phase I/II study on patients with colorectal cancer to examine the safety and efficacy of tumor draining lymph node derived T cell therapy (112).

In the first part of the study, T cells obtained from tumor draining lymph nodes were expanded *in vitro* for a median of 28.5 days (range 23–33 days). Cells cultures were monitored during expansion with flow cytometry. The phenotypes of the harvested cells before transferring back to the patients were measured. Enlarged proportion of CD3⁺CD8⁺ cytotoxic T lymphocytes was found ($p < 0.0001$) in the cell culture. No treatment-related toxicity was observed in this section. For the second part of this study, stage IV patients with advanced disseminated colon cancer were followed routinely according to local guidelines. We found that the 24-month survival rate in the T cell therapy group was significantly increased than in the control group: 55.6 versus 17.5 % ($p = 0.02$). The median overall survival of the T cell treated and control group was 28 and 14 months, respectively, however this result was not statistically significant ($p = 0.35$). Our study demonstrates that tumor draining lymph node based T cell immunotherapy is safe and feasible for postoperative CRC patients. In addition, this therapy may capable to improve the long-term survival of metastatic CRC without additional side effects. Further investigation of tumor draining lymph node based T cell immunotherapy is needed, preferably in a randomized controlled study comparing T cells therapy with best practice chemotherapy.

4.2 MICRO-METASTASIS DETECTABLE BY FLOW CYTOMETRY IN PATIENTS WITH PENILE CANCER (PAPER II)

When we considered investigating tumor antigen specific immune responses, virus associated malignancies was chosen for we could use viral proteins as one source of antigen for clonal T cell expansions. Thus, we decided to investigate penile cancer, where approximately half of the penile cancers are caused by an HPV infection (8). In order to examine whether the studies of immune responses in tumor draining lymph nodes will disturb the clinical staging process, based on our previous experience on micro-metastasis detection by flow cytometry (111, 113), we designed a pan-cytokeratin AE1/AE3 staining for detection of metastasis in lymph nodes from patients with penile cancer. Initially, we used one HPV cell line (HeLa cell line) with added PBMC to evaluate the sensitivity and stability of the assay. We found that as lowest as 0.1% of HeLa cells mixed with PBMC could be distinguished by flow cytometry. Furthermore, flow cytometry managed to detect HeLa cells remain the same sensitivity among different PBMC donors within 12h. Secondly we investigated 10 lymph nodes from 5 patients with pan-cytokeratin AE1/AE3 staining and FACS which we compared head to head with pathology results. In total, 4 LNs (4/10) (40%) were found pan-cytokeratin-positive from 3 of the 5 investigated patients by FACS. While routine pathology demonstrated metastasis in 3 LNs (3/17) from 2 out of 5 patients. Thus, flow cytometry detection suggested an upstaging of two patients.

There are multiple studies on improving the sensitivity in micro metastasis detection. In some studies, they used polymerase chain reaction (PCR) technique to detect the expression of tumor antigen mRNA (114, 115). Although these methodologies indeed improve the sensitivity, they are time consuming and can be difficult to apply in clinical routine. When using flow cytometry assay for micro metastasis detection, the total time

is approximately within 2 hours and easy to measure in an objective way and therefore a useful method to use for clinical routine detection of lymph node metastasis for staging.

4.3 PHENOTYPIC CHARACTERIZATION OF LYMPHOCYTES FROM TUMOR DRAINING LYMPH NODES FROM PATIENTS WITH PENILE CANCER (PAPER III)

There are few studies on penile cancer, especially with regards to immune responses, therefore we were interested to study the phenotype and the function of T lymphocytes in tumor draining lymph nodes from penile cancer patients. Single cell suspensions obtained from tumor, lymph nodes and peripheral blood were analyzed by FACS. The proportion of the different lymphocyte population in LNs varied between the patients. When we compared CD4/CD8 ratios between LN and peripheral blood, there was a significant difference ($p=0.0009$) comparing PBMCs and LNs where the CD4⁺/CD8⁺ ratio in PBMCs was 2.5 compared to 10 in LNs, demonstrating an expansion of CD4⁺ T lymphocytes in lymph nodes. When we investigated different lymphocyte populations, we found a significant correlation between CD8⁺ T lymphocytes and CD56⁺ expressing lymphocytes (Spearman $r=0.72$, $p<0.0001$). Furthermore, we found an inverse correlation between the fraction of CD19⁺ B cells and CD4⁺ T lymphocytes (Spearman $r=-0.61$, $p=0.0015$). Interestingly, we found an inverse correlation between the fraction of CD19⁺ B cells and the CD4⁺/CD8⁺ ratio (Spearman $r=-0.61$, $p<0.0001$). When we investigate patients, who died from penile cancer within two years from diagnosis, we found low CD4⁺/CD8⁺ ratios and/or low CD19⁺ B cells. Although very few patients altered balance in immune response may suggest immune suppression.

When analyzing CD4⁺/CD8⁺ ratios in lymph nodes comparing cells from HPV positive and negative patients, we found a significantly decreased ($p < 0.05$) CD4⁺/CD8⁺ ratio in lymph nodes derived from HPV positive patients suggesting that there was a relative increase in virus specific CD8⁺ T lymphocytes in HPV patients.

4.4 LYMPHOCYTE REACTIVITY TOWARDS TUMOR ANTIGEN AND VIRUS ANTIGEN (PAPER III)

First, we tested T cell responses against heat denatured tumor lysates. 13 available LNs from six patients were investigated with respect to blast responses towards the tumor extract by FASCIA. We observed CD4⁺ T lymphocytes blast transformation as a response in 6/13 LNs (46%) to tumor extract. Correspondingly, CD8⁺ T lymphocytes blast responses were seen in 7/13 (54%) LNs. In total we found blast responses indicating an immune response, either CD4⁺ or CD8⁺, in 9 out of 13 tested LNs by FASCIA using autologous tumor extract as antigen (69%) representing 5 out of 6 tested patients (83%). As nearly half of penile cancers are associated with HPV infection, we wanted to investigate if we could observe an immune response to HPV in the lymph nodes of HPV positive patients. We tested T-cell blast responses to Gardasil®, containing L1 antigen from HPV 6, 11, 16 and 18, in the FASCIA assay. Blast responses were observed in PBMCs and in LN cells of the HPV positive patient no. 8. Both HPV-positive patients (patient 8 and patient 9) responded to Gardasil® and to tumor extract. In a dose response assay, Gardasil® was added to the cell culture from 0.1-1 µg/ml and blast transformation was evaluated on day 7. The LN-derived lymphocytes from HPV-positive patient 9 responded in a dose dependent manner against Gardasil®. When we investigated the cell surface expression of the activation

marker HLA-DR, a 10-fold increase in the Gardasil stimulated group was found compared to control, suggesting Gardasil dependent T cell activation.

In a recent study using a cocktail of HPV E6 and E7 peptides to stimulate and expand Tumor-Infiltrating T cells (TIL) from cervical cancer patients, promising responses after adaptive cell therapy were demonstrated (52). In this study, two patients with cervical cancer received a single infusion of HPV specific –TILs resulting in complete responses. The pilot study using E6 and E7 directed T cell therapy in cervical cancer suggested the potential value of HPV specific immunotherapy. Besides, E6 and E7 act as oncoproteins associated with the tumor suppressors: p53 and pRB, respectively, and therefore play major roles in tumor establishment. In a recent study HPV-specific CD8+ and CD4+ T-cell responses against E6 and E7, proteins encoded by synthetic plasmids (VGX-3100), were found in patients with cervical cancer (116), suggesting a potential role of specific T cell therapy in HPV malignancies. Thus, the use of E6 and E7 peptides to select and expand HPV specific T cells for immunotherapy of patients with penile cancer may be useful.

There have been many clinical trials with vaccine strategies of patients with HPV associated cancer, with limited success. A study suggested the essential part is to overcome the local immune suppression (117). Another study found that 23 out of 37 primary tumors (62.2%) expressed PD-L1 from patients with penile cancer, and the expression level correlated with metastasis (118). Therefore, combining HPV specific T cell therapy with PD-L1 blockade may increase the response rate treating patients with penile cancer.

4.5 HPV INDUCED MHC CLASS I DOWNREGULATION

As one of the virus induced immune escape mechanisms, HPV could down regulate the MHC (HLA) Class I expression. Studies on early proteins including E2, E5 and E7 suggested they might involve in MHC downregulation (91, 92, 119).

We demonstrated transfection of overexpressed E2, E5 and E7 of HPV18 strain to HeLa cell lines. Down regulated MHC Class I expressions were found in E2 and E5 overexpression sets (Fig 5.). However, this is just one round with duplicates, more repeated experiments are warranted.

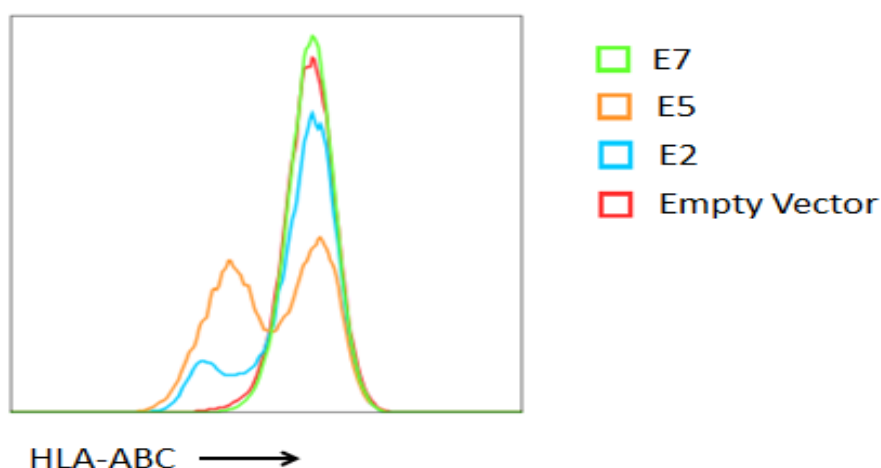


Fig 5. Expression of HLA-ABC on transfected HeLa cells

The seeded HeLa cells were transfected separately with DNAs which are coding E2, E5 and E7 from HPV18 strain and an empty vector as control (in duplicates). The transfected cells were harvested and stained with HLA-ABC antibody then detected by flow cytometry. A shift of expressing signal was observed in E2 and E5 transfected cells which suggest the downregulation of MHC I.

4.6 EFFECT OF DIFFERENT IL-2 FAMILY CYTOKINES ON ANTIGEN SPECIFIC T CELL EXPANSION

Several studies on IL-2 cytokine family suggested they could enhance T cell expansion. However, it is not clear which combination of cytokines is optimal for antigen specific T cell expansion. To study virus specific T cell responses we used CMV as a model system. We collected lymphocytes from peripheral blood from CMV positive donors and stimulated the cells with CMV peptide together with different combinations of cytokines chosen from the IL-2 cytokine family (IL-2, IL-7, IL-15, and IL-21) to study CMV specific T cells as a model. The experiments described below are initial setup attempts thus no strong conclusion could be made.

In our first attempt, we conducted 4 groups of cytokine combinations (IL-2 only group, IL-2 and IL-7 group, IL-2, IL-7 and IL-21 group and IL-2, IL-7 and IL-15 group) and recorded the number of the cells during the expansion and use the no antigen groups as controls. We found that all cytokine groups (with or without antigen) could induce CD4⁺ and CD8⁺ T cells expansion in early stage and the groups without antigen stimulation gradually stopped to expand on Day 35 except for the IL-2 and IL-7 combined group. While the groups stimulated by antigen from the beginning, the presence of specific antigen boosted the expansion dramatically after they were restimulated by the same CMV peptide. An increased expression of CD4⁺CD69⁺ and CD8⁺CD69⁺ T cells were found in all groups with CMV peptide which was not found in cytokine only groups which suggest the activation marker expressing cells were CMV specific T cells. Considering IL-2 could also trigger regulatory T cell expansion, similar experiments were conducted to test some other combinations without IL-2 which include: 1. IL-7 and IL-15; 2. IL-7 and IL-21; 3. IL-7, IL-15 and IL-21. We observed IL-7 and IL-21 group and the group combined IL-7, IL-15 and IL- 21 promote

CD4⁺ T cells expansion. These experiments gave us some valuable information of cytokine cocktails to be further analyzed with patient material.

A study demonstrated expansion of antigen specific T cells from naïve human CD8⁺ T cells with the help of initial IL-21 stimulation followed by a combination of IL-7 and IL-15 (109). It brought us to consider that the effect of different cytokine combinations may depend on the maturation status of the cells, i.e. naïve or memory T cells. We made several attempts to sort out memory T cells followed by testing with different cytokine combinations. After restimulation with CMV peptide, cells did not continue to expand and even became apoptotic, and we found no significant differences between cytokine groups. There may be several explanations for the results, since specific T cells could be sensitive in this phase for restimulation, IL-2 induced cell death, and therefore a proper time-point for restimulation needs to be worked out to optimize not only combinations and concentrations of cytokines.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Immune therapy has become a powerful weapon against cancer (120). As one of the cell therapies, our methodology which expands tumor specific T cells from tumor draining lymph nodes *ex vivo* and transfuses them back to the patients has demonstrated the flexibility and safety for patients with colon rectal cancer. Prolonged survival rate was found in stage IV patients who received T cell therapy compared with control groups ($p=0.02$), whereas no treatment related toxicity was observed. It suggested that our T cell therapy which obtains tumor specific T cells derived from tumor draining lymph nodes has a potential value for colorectal cancer treatment. Further clinical study is warranted.

In our T cell therapy, we used autologous tumor lysis as source of tumor antigen, which is an unknown factor that cannot be adjusted in expansion protocols. In order to study antigen specific T cell responses we considered to use known antigens such as viral antigens as source of stimulator. Penile cancer associated with HPV infection then became a good option to start with. Initially we needed to convince the pathologists that our methodology of using tumor draining lymph nodes is not disturbing clinical tumor staging. We found that staining with pan-cytokeratin AE1/AE3 detecting aberrant tumor cells by flow cytometry could obtain more information than standard pathology examination, since flow cytometry detected two additional lymph nodes with metastasis while reported negative by routine pathology examination.

Antigen specific T cell responses were studied by conducted T cell reactivity towards tumor lysis and HPV antigen for patients with penile cancer. A study demonstrated that L1-specific $CD8^+$ CTLs and E7-specific $CD8^+$ CTLs have the same effectivity in eliminating autologous HPV^+ tumor cells in patients with cervical cancer (88). We

decided to use the HPV vaccine (Gardasil) as an antigen source in our study. 12 patients with penile cancer were recruited in this study, and we found 5/6 tested patients (83%) demonstrated T cell recognition of tumor associated antigen(s). In HPV-positive patients, a dose dependent T-cell response against late HPV proteins, from the vaccine Gardasil®, was observed. Further studies on the immune reactivity towards virus antigens in this type of cancer are warranted.

Increased understanding of viral infected diseases, HPV could down regulate MHC Class I to escape the immune surveillance. Several studies found HPV E2, E5 and E7 were involved in MHC downregulation (91, 92, 119) in other solid cancers. It would be interesting to know whether the express of those early proteins will affect MHC Class I expression in penile cancer cases.

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