Adoptive T Cell Therapy for Treatment of Metastatic Melanoma

ARIAN SADEGHI
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjöldsväg 20, Uppsala, Tuesday, March 8, 2011 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Malignant melanoma is a common type of solid tumor that causes high cancer-related mortality in young adults of Northern Europe. The incidence of melanoma increases rapidly which renders us a special responsibility to investigate this disease in depth. One recent promising approach to treat malignant melanoma is adoptive cell therapy with tumor-directed autologous T cells. This thesis aims to improve this therapy in four different studies. We first sought to establish a protocol for the assessment of melanoma-specific T-cell cultures in order to screen for optimal specificity and reactivity in a robust, reliable and simple manner. The conclusion was that reactive cells could be found in a majority of patients and could be screened for specificity by stimulation with melanoma cell lines.

In the next study, 28 melanoma patients with advanced disease were treated with autologous tumor-infiltrating T cells. Objective responses (18%) including one sustained complete response were observed. This is the first study in cancer patients with autologous T cell transfer combined with low-dose s.c. IL-2 as supportive cytokine.

In the following two studies we wanted to improve management and culture conditions of the T cells. When investigating methods for improved handling and preservation of large numbers of T cells, we observed that freeze-thawing of T cells could impair the metabolic activity of the T cells. Another conclusion was that rapid expansion of T cells could lead to loss of antigenic specificity and apoptosis. These adverse effects could be prevented with short time recovery. In order to improve expansion methods, mass expansion of T cells in an automated bioreactor was evaluated. We concluded that the bioreactor is suitable for this task and allows for higher cell densities and absolute cell numbers compared to traditional culturing conditions without influencing cell phenotype or reactivity. Taken together, my current studies present guiding principles and encouragement for the further development of immunotherapies for treatment of patients with malignant melanoma.

Keywords: Malignant melanoma, Adoptive cell therapy

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urn:nbn:se:uu:diva-143698 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-143698)
Doctors are men who prescribe medicines of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing

Voltaire

To my Family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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*G.U. and A.S. contributed equally to this paper*
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<th>Description</th>
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<tbody>
<tr>
<td>ACT</td>
<td>Adoptive cell transfer</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 Ligand</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CDKN2a</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
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<tr>
<td>CHK1</td>
<td>Checkpoint kinase 1 homologue</td>
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<tr>
<td>CM</td>
<td>Complete medium</td>
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<tr>
<td>CR</td>
<td>Complete response</td>
</tr>
<tr>
<td>cSMAc</td>
<td>Central supramolecular activation cluster</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<td>HSP</td>
<td>Heat-shock protein</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LFA1</td>
<td>Leukocyte function associated antigen 1</td>
</tr>
<tr>
<td>LMP</td>
<td>Low molecular mass protein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LRR</td>
<td>Leucin-rich repeats</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC1R</td>
<td>Melanocortin receptor 1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NKDC</td>
<td>Natural killer dendritic cell</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PD</td>
<td>Programmed death</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death receptor 1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death ligand 1</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PR</td>
<td>Partial response</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>pSMAC</td>
<td>Peripheral supramolecular activation cluster</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response evaluation criteria in solid tumors</td>
</tr>
<tr>
<td>RGP</td>
<td>Radial-growth phase</td>
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<tr>
<td>SD</td>
<td>Stable disease</td>
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<tr>
<td>TA</td>
<td>Tumor antigen</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor associated antigen</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TGF(\beta)</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor-infiltrating lymphocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor related apoptosis inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>VGP</td>
<td>Vertical-growth phase</td>
</tr>
<tr>
<td>(\alpha)-MSH</td>
<td>(\alpha)-melanocyte-stimulating hormone</td>
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</table>
Aims of the study

The studies presented within this thesis were aimed towards two general goals. The first goal was the isolation and characterization of optimal tumor specific T cells and subsequent therapy of malignant melanoma using these cells. Cellular therapies with \textit{ex vivo} expanded antigen-specific autologous T cells have proven to be promising. In paper I, we sought to establish a reliable and simple method for generation and analysis of tumor-specific T cells. In paper II, we evaluated the results of a pilot trial wherein we attempted adoptive T cell therapy in 28 stage IV melanoma patients who had progressed on standard treatments.

Our second goal was improvement of methods in handling and management of T cells during cultivation, expansion and preservation. In paper III, we analyzed the impact of \textit{ex vivo} culture, clonal expansion and cryopreservation of the cells. In paper IV we evaluated the expansion of T cells in the WAVE bioreactor and compared this with the traditional methods of cell expansion.
1. Introduction

The mammalian immune system is a dynamic and complex machinery composed of many cell types that interact with each other and with non-immune cells. The main functions of the immune system is distinction between self and non-self, protection against infectious invaders, elimination of damaged cells and monitoring of tissue homeostasis. Immune systems are found within almost all organisms and have been fine-tuned throughout the course of evolution. Therefore, it may seem surprising that humans, who are equipped with a refined and time-tested defense mechanism, are still afflicted by cancer at such a high frequency. Cancer originates from mutations that alter crucial pathways regulating tissue homeostasis, cell survival and cell death. In recent decades we have learned that cancers are composed of a multitude of cell types, such as fibroblasts, endothelial cells, immune cells and cells that form vasculature. Cancer development is enhanced as tumor cells harness the capability to promote their own survival. Today we have recognized that each stage of cancer development is susceptible to regulation by immune cells and that full activation of adaptive immune cells may result in eradication of tumor. However, cancer is an insidious disease with the ability to escape surveillance by the immune system and to disrupt the physiological homeostatic balance of the host. In order to combat this, the balance must be skewed in favor of the immune system and stimulation of immune responses and this can be accomplished through various immunotherapeutical approaches. The field of immunotherapy has today evolved into many varieties that can generally be divided in to active immunization approaches involving vaccination or passive immunotherapy involving monoclonal antibodies or adoptive transfer of immune cells.
1.1 Adoptive immunotherapy of cancer

Since the 1950s, when it was first demonstrated that transferred immune cells could mediate anti-tumor effects in animals, immunotherapy has captured the imagination of scientists. The field progressed from early demonstrations of anti-tumor effects of transferred leukocytes to clear demonstrations of complete regression of established tumors in animals\textsuperscript{1,2}. Soon after, in the 1960s, splenocytes obtained at splenectomy in non-tumor-bearing individuals were transferred to cancer patients, but with no specific benefit\textsuperscript{3}. Investigators reasoned that prior immunization of leukocyte donors with tumor cells might enhance tumor response and in some of these studies objective clinical responses were observed, particularly in patients with melanoma\textsuperscript{4-6}. However, generation of large numbers of allogeneic immune cells was met with technical difficulties and safety concerns. In the late 1970s, the first attempts at deriving autologous lymphocytes were made initially with conditioned media\textsuperscript{7,8} and eventually with purified and recombinant IL-2\textsuperscript{9}. Following the demonstration of \textit{in vivo} tumor inhibition with transferred cells in mice, studies were performed in patients using autologous cells\textsuperscript{10}. Safety of treatment was demonstrated and some responses were observed, especially in virus-induced malignancies and disorders associated with Epstein-Barr virus and Cytomegalovirus\textsuperscript{11-13}.

Today the field has progressed significantly by using a combination of different approaches to isolate and expand optimal cells and to support transferred cells \textit{in vivo} in order to enhance adoptive cell therapy. This therapy has today the power to eradicate advanced and disseminated tumors that are refractory to other conventional therapies. With our advancing knowledge of tumor biology and immunology, together with enhancements in medical technologies, further opportunities for refinement of adoptive immunotherapy have evolved (Figure 1). This thesis highlights the research and approaches aimed at improving adoptive cell transfer immunotherapy for treatment of malignant melanoma.
Figure 1. **Schematic overview of adoptive immunotherapy.** The central process of adoptive immunotherapy involves the isolation of immune cells (e.g. T cells, NK cells) from patients, followed by *ex vivo* activation and expansion. Expanded cells are transferred back to the patient after preconditioning chemotherapy. Cytokines can induce proliferation of *ex vivo* expanding cells and also maintain cell homeostasis after cell transfer. The tumor microenvironment can be manipulated with e.g. antibodies before cell transfer in order to augment the anti-tumor effect of transferred cells.

1.2 Innate and adaptive immunity

With advancing knowledge about our immune system, the more amazed we are by its complexity. One has to start with the evolutionary history of innate and adaptive immunity in order to understand how our immune system functions and sometimes dysfunctions.

Innate immunity is an evolutionary ancient mechanism of host defense and has been crucial in the competitive struggle for existence. It can be found in species at almost every evolutionary level. This is because the earth’s biomass consists primarily of microorganisms, many of which are pathogenic. Although innate immunity is remarkably diverse, a unifying characteristic is the use of germline-encoded pattern recognition receptors for pathogens or damaged self components, e.g. Toll-like receptors (TLR) or nucleotide-binding domain leucin-rich repeats (LRR)- containing receptors. Innate immunity lies behind most inflammatory responses mediated by
non-specific effector cells such as macrophages, polymorphonuclear leukocytes, mast cells and NK cells. Adaptive immunity appeared in vertebrates around 500 million years ago coinciding with the appearance of functional organ changes including the development of the lower jaw\textsuperscript{15}. It consists of clonally diverse lymphocytes with the potential of self-renewal and armed with a unique antigen receptor used to trigger its activation. Antigen-activated lymphocytes undergo clonal expansion and differentiate to mature lymphocytes with effector/memory or regulatory functions (T cells) or into plasma cells producing antibodies (B cells). Effective cellular responses in B and T cells are dependent upon the participation of different cells of the innate immunity like various phagocytic cells, dendritic cells (DC) and NK cells. The adaptive immune system lacks the perfect self-nonself discrimination of innate immunity that has been selected over evolutionary time. Distinction of self-nonself in adaptive immunity is selected in individual somatic cells during thymic maturation. Upon entering the thymus, precursors of T cells originating from the bone marrow express TCRs and CD4 and CD8 coreceptors. Selection processes within the thymus eliminate self-reactive T cells and promote survival of T cells that bind self-antigens with low affinity. However in some instances these selection processes fail, leading to T cells directed towards self-antigens which in turn may lead to autoimmunity. This lack of discrimination, although in most cases afflicting and harmful, may in some instances be advantageous for us. For instance, most cancer cells are normal somatic cells that due to genetic abnormalities display uncontrolled growth. Cancer cells are like any other self-cells tolerized by the immune system and permitted to grow. However, if this self-tolerance is broken, then the immune system can attack the self-antigen expressing cancer cells and eradicate or control the growth of the tumor.

1.3 Antigen presentation

One of the fundamental processes inducing and regulating immune responses is the establishment of cell-cell contacts between T cells and antigen presenting cells (APC) such as DC or macrophages. Antigen recognition in the peptide-binding groove of cell surface expressed major histocompatibility complex (MHC) class I and class II molecules by specific T cells is crucial for T cell activation. Such contacts elicit a signal transduction leading to various outcomes, including the maturation of the T cell, the generation of effector, memory or regulatory T cells and the cytotoxic killing of target cells. Initial contact between T cells and APC leads to activation of transcription of specific genes, recruitment of cell-surface receptors and morphological changes. The interaction of T cell-APC at single cell level consists of three phases: contact acquisition, formation of immunological synapse and after minutes to hours, detachment and migration\textsuperscript{16}. Acquisition of peptide
antigen by the two main classes of MHC molecules is executed by to different pathways.

MHC class I molecules report on intracellular events such as intracellular viral or bacterial infections or cellular transformations. The MHC class I molecule, known as human leukocyte antigen (HLA) A, B and C in humans, consists of 2 polypeptide chains, α and β2-microglobulin (b2m). In the MHC class I pathway, proteins from e.g. virus in the cytosol are proteolytically degraded and transported to the ER via transporter associated with antigen processing (TAP) proteins. Peptides that are 8-12 amino acids long are bound to the peptide-binding cleft of the MHC molecules. After assembly, the complex is transported via the Golgi and displayed on the cell surface to CD8⁺ T cells.

MHC class II molecules present samples of the extracellular milieu to CD4⁺ cells. This molecule, known as HLA DR, DP and DQ in humans, consists of α and β chains and is present on APCs such as dendritic cells, macrophages and B cells. In the class II pathway, exogenous peptides are taken up through phagocytosis. Engagement of TLRs on the cell surface or within the cytosol early in the course of phagosome formation activates T cells more potently than antigen alone. The antigens are processed within the phagosome and are typically 9-25 amino acids long. Newly biosynthesized MHC molecules leave the ER in vesicles and fuse with antigen containing phagosomes where MHC and antigen complexes are formed. Peptide loaded MHC molecules are transported through vesicles and presented on the cell surface.

Extracellular antigens can be presented to CD8⁺ T cells through MHC I by cross presentation which is required for defense against many viruses and tumors. Self-antigens are also presented by this pathway in a process named cross-tolerance.

MHC class I and II molecules are presented at low levels on the surface of monocytes and naïve B cells. The expression of these molecules is strongly upregulated after maturation. Immature DCs express intermittent levels of MHC molecules, maturation following ligation of TLRs or CD40 or stimulation with cytokines like interferon gamma (IFNγ) upregulate and stabilize MHC expression up to 10-fold.

1.3.1 Antigen presenting cells

Dendritic cells, B cells, monocytes and macrophages express a variety of cell surface molecules that are involved in T cell binding and activation. Important molecules on these cells are the intercellular adhesion molecule 1 (ICAM1) which stabilizes the immunological synapse by binding to leukocyte function associated antigen 1 (LFA1) on T cells and CD80/CD86 that
control T cell activation through interaction with CD28 or cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed by the T cells.

**Figure 2. Diversity of molecular interaction at the immunological synapse.** The peptide/MHC complex and TCR interaction provide the first signal for T cells activation. Adhesion molecules such as LFA and ICAM strengthen synapse formation and interaction between T cell and APC. CD4 and CD8 together with CD3 and the ζ-chain modify the signal. CD80/CD86 interaction on the APC with CD28 on the T cell provides the secondary signal to activate the T cells. CTLA-4 on activated T cells has higher affinity towards CD80/CD86 and delivers an inhibitory signal to the T cell.

### 1.4 T Lymphocytes

T cells are a heterogeneous population of cells that are hierarchically organized and display different maturation and differentiation states and different capacities for clonal expansion and self-renewal. All lymphocytes originate from bone marrow stem cells and T cells undergo maturation in the thymus where they express antigen receptors together with CD4 or CD8 co-receptors.

#### 1.4.1 CD4+ T cells

CD4+ T cells recognize MHC class II peptide complexes and differentiate into different T helper (T<sub>H</sub>) effector subsets that can be distinguished through cytokine phenotypes. T<sub>H1</sub> cells produce IFN<sub>γ</sub> and tumor necrosis factor alpha (TNFα) and mediate protection against intracellular pathogens. They also contribute to CD8+ cytotoxic T lymphocyte (CTL) activation through production of IL-2 and stimulation of APCs via CD40L/CD40 interaction, which in turn produce IL-12 for enhanced T cell development. T<sub>H2</sub> cells produce interleukin 4 (IL-4), IL-13 and IL-25 and mediate protection against extracellular pathogens and contribute to B cell activation. T<sub>H17</sub> cells pro-
duce IL-17 and IL-22 and are thought to clear extracellular pathogens that Th1 and Th2 are unable to handle. T regulatory cells (T_{reg}) are CD4+ T cells which produce inhibitory cytokines like IL-10 and Tumor growth factor beta (TGF\(\beta\)) and suppress effector cells like CTLs, NK cells, B cells and APCs. Their function is induction of peripheral tolerance. Presence of different cytokines shapes the differentiation of naïve CD4+ T cells into the different Th subsets. IFN\(\gamma\) stimulates the induction of the Th1 arm while IL-4 induces the Th2 arm. TGF\(\beta\) converts naïve T cells into T_{reg} and into Th17 subset in presence of IL-6 \(^{23,24}\) (Figure 3).

1.4.2 CD8+ T cells

CD8+ cells can differentiate to CTLs and become the rapid destroyer cells in the immune system. In order to become CTLs these cells require the primary antigen-specific signal through TCR interaction with MHC I peptide complex and secondary costimulatory signals mediated through CD28 molecules on the T cells and B7 molecules on activated APCs. They perform a surveillance function that involves recognition and termination of virus-infected and potentially malignant cells. Upon activation CTLs have the ability to proliferate and produce IL-2 in an autocrine and paracrine fashion. After a few days however, CTLs start entering a state of anergy and exogenous IL-2 must be provided for continued proliferation. TCR recognition of target MHC I-peptide complex causes the CTLs to form an immunological synapse with the target cell. CTLs will at this stage regain their ability to produce IL-2 and respond until the source of antigen diminishes \(^{25}\). Cells that express peptides derived from mutant cellular proteins or oncogenic viral proteins that are presented on MHC class I are potential CTL targets. Lytic agents are delivered into the immunological synapse between CTLs and target cells and target cells will be destroyed. These lytic agents are the pore-forming protein perforin, granzymes and proteases that cause rapid cell death through cleavage of different substrates \(^{26}\).
Figure 3. Differentiation of helper T cells subsets. Following activation by APC and depending on the local cytokine environment, naïve CD4⁺ cells differentiate into different effector T cell subsets. These cells produce different cytokines and have distinct immunoregulatory functions. IFNγ produced by TH1 cells is important for the regulation of antigen presentation. IL-4, -5 and -13 regulate B cell responses. IL-17, -21 and -22 regulate inflammatory responses. TGFβ and IL-10 suppress immune responses.

Memory T cells can be either CD8⁺ or CD4⁺ and originate from both TH and CTL subsets. These long lived lymphocytes mediate extended anti-tumor and anti-viral memory and are divided into central (T_cm) and effector (T_em) memory cells depending on their phenotype and cell surface receptor profile. Effector memory cells are found in non-lymphoid tissue and carry out immediate effector functions through secretion of IFNγ or IL-4. Central memory cells are long-lived and provide long-term protection and have the ability to home to lymph nodes and produce IL-2 upon antigen recognition ²⁶.

T cells in different maturation stages display variations in expression of receptors involved in cell-cell communication. During thymic maturation, T cells express and upregulate TCR, CD4 and CD8 molecules together with integrins (LFA1) and other adhesion molecules, most of which are involved in stabilization of the cell-cell junction such as CD44. In circulation and upon primary activation, naïve T cells further upregulate the expression of these molecules together with additional molecules like CD2 and CD28 ²⁷. As the T cells mature the levels and diversity of adhesion molecules increases. Overactivation of T cells and autoreactivity is prevented by upregulation
of inhibitory receptors like programmed death (PD) receptors and CTLA-4 on the surface of the T cells and their cognate ligands on APCs or tumor cells (Figure 2). The TCR together with CD28/CTLA-4, CD2, CD3 and CD4/CD8 form a central supramolecular activation cluster (cSMAC), while the adhesion molecules form a peripheral SMAC (pSMAC) needed for a stable molecular synapse.

After thymic selection, recirculating naïve T cells maintain their homeostasis through constitutive weak and unspecific interaction with DCs and other APCs as they migrate through the perifollicular regions of the lymph node. Such repetitive interactions are thought to provide sub-threshold survival signals for the T cells in the absence of activation and clonal expansion. During inflammation, multiple APCs residing in the lymph node or immigrated from peripheral tissue present a similar antigen repertoire and provide serial pulses of signals that enable the T cells to overcome the threshold for activation. Naïve and pre-activated T cells require a stable connection with APC and antigen-mediated TCR signaling to reach the threshold for activation or differentiation in to e.g. IL-2 secreting TH cells.

During interactions between T cells and APCs or other cells, secretory functions of T cells and exocytosis of soluble factors is achieved through polarization of intracellular vesicles toward the cell membrane. Released compounds are accumulated between the membranes of each cell, thereby reaching high local concentrations. For CTLs, very low antigen densities on the surface of target cells are required for cytolytic activity. At low doses of antigen, FAS (CD95 and CD95L interaction) –mediated killing occurs which is independent of the stability of the immunological synapse. Higher doses of antigen and a stable cell-cell contact seems to be required for the polarized release of granzymes and perforins from the secretory pathway. In activated CD4+ TH cells, intracellular vesicles containing cytokines become polarized toward the cell-cell junction in a unipolar or multipolar fashion. It has been shown that TH cells sense and respond to different antigen loads and that there is a preference for the APCs with the highest antigen load. In addition to CD4+ and CD8+ T cells that express αβ TCR, γδ T cells express γδ TCR chains and are CD4−CD8−. γδ T cells mediate cytolytic activity, respond to IL-2,3 and 4 and recognize unconventional antigens that remain unidentified by αβ T cells.
2. Tumor immunology

It is well established that tumorigenesis in humans is a multistep process of genetic alterations that drive the progressive transformation of normal cells into malignant ones. This process is analogous to Darwinian evolution, in which a succession of genetic changes, conferring growth advantages, leads to progressive conversion of normal cells to malignant variants\textsuperscript{35,36}.

Virtually all mammalian cells, including cancer cells, are governed by a similar molecular principle regulating proliferation, differentiation and death. However in cancer cells, six essential cell physiological alterations dictate malignant growth and these are summarized as: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative status, sustained angiogenesis and tissue invasion and metastasis\textsuperscript{37}. These physiological alterations are at each step subjected to control by cell extrinsic i.e. the immune system (immuno-surveillance) and to cell intrinsic anti-cancer defense mechanisms.

2.1 The concept of cancer immunoediting

The immune system can recognize the intermediate steps in a process through which normal cells progressively transform from premalignant precursors into invasive cancers. The immune system can interfere with this process and destroy the precursor cells before they become clinically apparent in a process that is called immunosurveillance\textsuperscript{38}. Although cancer cells induce immune responses, cancer can still develop. This means that the immune system is sometimes inefficient in preventing development of cancer due to immunoselection of tumor cells that evade the immune system or because tumor antigen-specific immune tolerance is induced\textsuperscript{39}. The process of cancer immunoediting consists of three phases: Elimination (Protection), equilibrium and escape (Figure 4). Elimination of tumor begins with several cell intrinsic defense pathways that involve proteins such as P53, retinoblastoma protein (Rb) and checkpoint kinase 1 homologue (CHK1) that protect cells from oncogenic or genotoxic stimuli that may lead to malignancy\textsuperscript{40}. During tumor growth, innate and adaptive immunity, alerted by danger signals, start to actively kill the transformed cells through a range of different cell types and effector molecules. The most prominent of these are the CD8\textsuperscript{+} CTLs that kill stromal and tumor cells in an MHC class I restricted manner.
In numerous cancers, the presence of tumor infiltrating T lymphocytes (TILs) is an important prognostic marker. In malignant melanoma in particular, there is a direct correlation between the number of TILs and prolongation of survival and development of metastatic disease. Activated CD4+ cells recognize and convert tumor infiltrating IL-10 producing M1 macrophages into IFNγ producing M2 macrophages. TH1 cells also release IFNy which has an anti angiogenic effect.

Figure 4. Cancer immunoediting. The process of Immunoediting consists of three stages: elimination, equilibrium and escape. Cancer immunosurveillance starts with the recognition of transformed cells by the innate and adaptive immune system. Initially, different danger signals attract cells of innate immunity such as NK cells and macrophages. In addition CD4+ and CD8+ T cells recognize tumor specific or tumor associated antigens in a MHC I or II restricted manner. Recognition by innate and adaptive immunity leads to direct killing of transformed cells, production of effector molecules that facilitate killing and to formation of memory cells. During the progression into the second phase the persisting tumor cells are prevented by the pressure from the immune system to expand. The escape phase begins when the balance between the immune system and tumor is tilted toward tumor growth. This can be due to several factors such as exhaustion of the immune system, emergence of tumor cells variants (shown in brown) or tolerance towards tumor antigens. Non-immunogenic transformed cells have the ability to directly enter the escape phase. Figure adapted from Schreiber et al. Nature Volume 6, November 2006.
Th2 cells produce IL-4 which activates humoral immunity and also blocks neo-angiogenesis indirectly through an effect on stromal cells. Other important cells are NK and Natural killer T (NKT) cells that can lyse tumor cells through a MHC-unrestricted, tumor necrosis factor related apoptosis inducing ligand (TRAIL) or perforin-dependent pathway. Tumor-specific antibodies can exert tumorcidal or antiproliferative effects directly but may also induce complement-mediated lysis or attract NK cells and macrophages expressing receptors for IgG. In the next phase of immunoediting, residual cancer cells that are highly mutable persist and survive and create a period of transition to the third phase of cancer-immunoediting, escape. In this phase, the pressure from the immune system has influenced and reshaped the immunogenic quality of the tumors that grow. Cancer cells that have averted immune responses and circumvented the immunological suppression or that have directly attenuated the antitumor functions of the immune effectors grow progressively.

2.2 Mechanisms of tumor escape

As a result of the selective pressure exerted by the immune system, tumors have developed a series of strategies to evade elimination. One common strategy is downregulation or loss of HLA class I expression or of molecules that are involved in antigen processing and presentation such as TAP or low-molecular-mass proteins (LMPs). This is common in melanomas, epithelial cell cancers, lung cancers and colorectal carcinoma. Tumors may also develop resistance to CTL-mediated killing by blocking the granzyme B and perforin pathways through overexpression of serine-protease inhibitor P19. In addition, downregulation or mutation of death receptors, methylation or mutation of the gene encoding caspase-8, overexpression of caspase-8 (FLICE)-like inhibitory protein (FLIP) or expression of decoy receptors for TRAIL also cause resistance to CTL-induced lysis. Several tumors or tumor-associated myeloid cells are actively involved in suppression of immune response through overproduction of nitric oxide and increased arginase-1 activity which can inhibit T cell function. Many tumors, particularly colon, prostate and pancreatic carcinomas produce indoleamine 2,3-dioxygenase (IDO) which blocks proliferation of CD8+ cells and promotes apoptosis of CD4+ cells at the tumor site. Tumor cells can also, by expressing CD95L, induce apoptosis in CD95 expressing tumor-specific T cells. It is common for the immune system to develop tolerance towards the tumor cells and for initially avid CD4+ and CD8+ cells to lose antitumor activity at the later stages of tumor expansion. Usually in the later stages of tumor expansion there is an increase in Treg and myeloid suppressor cell that contribute to reduction in the immune responses. Another strategy of immune escape is to manipulate the tumor microenvironment in order to inhibit
maturation, differentiation and function of antigen presenting cells. By producing various factors such as IL-6, IL-10, TGFβ, nitric oxide synthase 2 (NOS2) and IDO the tumor create dysfunctional (tolerogenic) APCs that mediate immunosuppressive effects and promote T_{reg} differentiation\textsuperscript{58,59}. Paradoxically, innate and adaptive immunity can also contribute to tumor growth through promotion of angiogenesis and tissue remodeling by production of growth factors, cytokines and matrix metalloproteinases\textsuperscript{60}.

2.3 Danger signals

During the recent decades, two models describing how immune responses are initiated have been introduced: the stranger and the danger models. In the stranger model, introduced by Janeway\textsuperscript{61}, antigen presenting cells expressing pattern-recognition receptors (PRRs) recognize the unique features of pathogen-associated molecular patterns (PAMPs) of microbial molecules. PAMPs activated APCs migrate to lymphoid tissues and present antigen and co-stimulatory molecules to T cells. In the danger model proposed by Matzinger\textsuperscript{62}, the immune response is initiated by molecules know as damage-associated molecular patterns (DAMPs) produced by stressed, damaged, apoptic or necrotic cells and tissues. Examples of DAMPs that function as adjuvants are heat-shock proteins (HSPs), uric acid, high-mobility group box 1 (HMGB1) and genomic double-stranded DNA\textsuperscript{63-65}. The cognate receptors for DAMPs on APCs are mainly TLRs (TLR2, 4 and 9), integrins and chemokine receptors\textsuperscript{66,67}. 


3. Malignant Melanoma

3.1 Epidemiology and risk factors

Cutaneous tumors are the third most common human malignancy with basal cell carcinoma, squamous cell carcinoma and melanoma being the most common forms. Of the estimated 2-3 million annual cases of skin cancer worldwide, malignant melanoma accounts for 132,000 cases, resulting in 66,000 related deaths (World Health Organization). According to the Swedish National Board of Health and Welfare, about 2,300 new cases are reported each year leading to 400 melanoma related mortalities in Sweden. The incidence of metastatic melanoma has increased over the past three decades with increasing death rates when compared with most cancers. The median age of diagnosis is 59 and the median age of death is 68 years of age. Metastatic and refractory melanoma has a poor prognosis with a median survival rate of 6 months and a 5-year survival rate of less than 5%. Melanoma tumorigenesis is caused by uncontrolled growth of melanocytes. Melanocytes are specialized pigmented cells originating from highly motile neural-crest progenitors that migrate to skin during embryonic development. Melanocytes are found predominantly in the basal levels of epidermis and the eye, producing melanin, the pigment responsible for skin and hair color. Homeostasis of melanocytes is regulated by keratinocytes which in response to UV radiation secrete factors that stimulate melanocyte proliferation, differentiation, motility and melanin production that ensues in the tanning of the skin. In malignant melanoma, cutaneous tumors stands for about 92% of the cases, other sites include mucosal membrane and ocular melanoma.

The risk of developing melanoma is enhanced with a family history of melanoma and multiple benign or atypical nevi. Different phenotypic characteristics and environmental factors like sun sensitivity or exposure to UV radiation are additional risk factors. Approximately, 25 to 40 percent of the members of melanoma-prone family members have mutations in the tumor-suppressor genes cyclin-dependent kinase inhibitor 2A (CDKN2a) and cyclin-dependent kinase 4 (CDK4). Ultraviolet radiation causes genetic changes in the skin, alters cutaneous immune function and induces production of DNA-damaging reactive oxygen species that affect keratinocytes and melanocytes. Exposure to UV radiation increases melanin production by
melanocytes through production and binding of α-melanocyte-stimulating hormone (α-MSH) to the melanocortin receptor 1 (MC1R). Light-skinned and redheaded individuals often carry germ-line polymorphisms in the MC1R gene\textsuperscript{76} that reduce the activity of the receptor, thus increasing the risk of melanoma considerably\textsuperscript{77}.

3.2 Pathology

The histological changes that describe the progressive changes of normal melanocytes to malignant melanoma are described by Figure 5. The first change in melanocytes is the development of benign nevi. Nevi rarely progress to cancer and most nevi cease proliferation and remain static for decades probably due to oncogene-induced cell senescence\textsuperscript{78}. Activation of the mitogen-activated protein kinase (MAPK)\textsuperscript{79} signaling pathway as a result of somatic mutations of N-RAS (15 - 30% of melanomas) or BRAF (50 - 70% of melanomas) stimulates growth in melanoma cells\textsuperscript{80,81}. Aberrant cell proliferation of nested melanocytes and the development of cytologic atypia in benign nevus or in new locations is the first step towards melanoma. This is reflected by lesions within the CDKN2A and phosphatase and tensin homologue (PTEN) pathways. Next follows an intradermal radial-growth phase (RGP) during which the cells start to proliferate intradermally. This is followed by a vertical-growth phase (VGP) where the cells have metastatic potential with clusters of cells invading the dermis and form an expansile nodule. Later stages of melanoma progression are notable for fundamental changes in the control of cell adhesion contributing to tumor invasion, tumor-stroma interactions and tumor-cell signaling\textsuperscript{73} (Table 1). The final step is successful spread and proliferation of cells in other areas of the skin and to distant organs where they can successfully proliferate and establish metastatic focus\textsuperscript{73} (Figure 5). Staging of cancer progression is based on depth of invasion into dermis, using either Clark’s model\textsuperscript{82} based on anatomic skin markers or Breslow’s model\textsuperscript{83} based on direct measurement of the depth of invasion from the epidermis. Stage I and stage II melanomas are localized primary tumors. Stage III is characterized by regional spread to the lymphatic system and in stage IV, metastases have disseminated to distant organs.
Table 1.

Genetic alterations during progression of malignant melanoma

<table>
<thead>
<tr>
<th>Gene Type</th>
<th>Gene</th>
<th>Frequency (%) and type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncogenes</td>
<td>BRAF</td>
<td>50-70% mutated</td>
</tr>
<tr>
<td></td>
<td>N-RAS</td>
<td>15-30% mutated</td>
</tr>
<tr>
<td></td>
<td>AKT3</td>
<td>Overexpressed</td>
</tr>
<tr>
<td>Tumor Suppressors</td>
<td>CDKN2A</td>
<td>30-70% deleted or mutated</td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>5-20% deleted or mutated</td>
</tr>
<tr>
<td></td>
<td>P53</td>
<td>10% lost or mutated</td>
</tr>
<tr>
<td>Others</td>
<td>Cyclin D1</td>
<td>6-44% amplified</td>
</tr>
<tr>
<td></td>
<td>MITF</td>
<td>10-16% amplified</td>
</tr>
</tbody>
</table>

Figure 5. Progression of melanocytes to malignant melanoma. There are 4 distinct states of melanocyte progression where each melanocyte clone gains advantage over surrounding tissue. Early benign nevus becomes dysplastic with morphologically atypical melanocytes. This is followed by a radial-growth-phase and a secondary vertical growth phase which is the first stage to have malignant potential. In the final stage of malignant melanoma the tumor cells have infiltrated vascular and lymphatic systems and spread to distant organs. Figure adapted from Marais et al, Nature Volume 445, February 2007

3.3 Melanoma antigens

Melanoma is immunogenic and lymphocytic infiltration during the vertical-growth phase has been observed in 10-20% of cases. In rare cases of spontaneous regression of melanoma, examinations have revealed immunomeditated tumor destruction by infiltrating lymphocytes, plasma cells and macrophages. Melanoma antigens can be classified according to Table 2. Being immunogenic, melanoma produces a plethora of different antigens which when
associated with HLA I molecules, are presented to the immune system. Some of these antigens are tumor specific and are produced by either mutational events or encoded by cancer-germline genes. What makes melanoma exceptional is that T cell responses have been observed against differentiation antigens present on normal melanocytes as well as on melanoma. The most prominent differentiation antigens are Melan-A/Mart-1, Tyrosinase and gp100. A very small fraction of T cells with naïve phenotype that are specific against these antigens and Melan-A/Mart-1 in particular have been found in healthy individuals. This fraction of Melan-A/Mart-1 specific CTLs is increased by 20-fold in melanoma patients. In advanced patients, a significant number of the Melan-A specific T cells have a memory effector phenotype in contrast to the naïve phenotype in healthy individuals. Infiltration of CTLs specific for various melanoma specific or associated antigens in the tumor is correlated to long term survival of the patients and a key factor to successful immunotherapy.

Table 2.

<table>
<thead>
<tr>
<th>Human melanoma antigens</th>
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</thead>
<tbody>
<tr>
<td>Melanocyte lineage or differentiation antigens</td>
</tr>
<tr>
<td>gp75 (TRP-1)</td>
</tr>
<tr>
<td>gp100</td>
</tr>
<tr>
<td>Melan A / MART-1</td>
</tr>
<tr>
<td>TRP-2</td>
</tr>
<tr>
<td>Oncofetal / cancer-testis antigens</td>
</tr>
<tr>
<td>Bage family</td>
</tr>
<tr>
<td>Gage family</td>
</tr>
<tr>
<td>NY-ESO-1</td>
</tr>
<tr>
<td>Tumor specific antigens</td>
</tr>
<tr>
<td>β-catenin</td>
</tr>
</tbody>
</table>

3.4 Treatment

Assessment of changes in tumor burden is fundamental for clinical evaluation of cancer therapeutics and both tumor shrinkage (objective response) and disease progression are important endpoints in clinical trials. Since the introduction of response evaluation criteria in solid tumors (RECIST) in 2000 and the revised RECIST 1.1 in 2009, many investigators, industry and government authorities have adopted these criteria in assessment of treatment outcomes. RECIST is based on radiological measurements of lesion size and can be summarized as: complete response (CR) - disappearance of all tumor foci; partial response (PR) – a reduction of at least 30% in tu-
mor diameters; stable disease (SD) – neither partial response nor progressive disease; and progressive disease (PD) - at least a 20% increase in the sum of all tumor dimensions from the smallest tumor size or the appearance of a new tumor lesion. The term objective response refers to either CR or PR.

3.4.1 Surgery and chemotherapy

Melanoma that is confined to the skin or lymph nodes is when detected early primarily treated by surgery. Some of these patients, particularly those with thin and nonulcerated lesions are cured by surgery alone \(^9^9\).

Melanoma is notoriously resistant to chemotherapy and single-agent chemotherapy results in limited (< 25%) response rates (Table 3).

Table 3.
| Response rates of treatments of malignant melanoma with single chemotherapeutical agents |
|---------------------------------|-----------------|-----------------------------|
| **Agent**                      | **Response rate (%)** | **Ref**                    |
| Dacarbazine                    | 7-25             | Bedikian 2006               |
| Temozolomide                   | 21               | Bleehen 1997                |
| Cisplatin                      | 16               | Glover 2003                 |
| Carboplatin                    | 19               | Evans 1987                  |
| Vindesine                      | 20               | Quagliana 1984              |
| Fotemustine                    | 16               | Avril 2004                  |
| Taxol                          | 16               | Bedikian 2004               |
| Carmustine                     | 20               | Jones 1992                  |

Dacarbazine is the only chemotherapeutic drug approved by the US food and drug administration for treatment of melanoma \(^9^0\). It has produced response rates between 7 and 25 %, with median duration of 5 to 6 months and complete responses of < 5% \(^9^1\). In order to improve response rates, combinatorial chemotherapies have been tested in clinical studies. The most effective multi agent combinations have included dacarbazine and other classes of chemotherapy agents (platinum compounds or alkaloids) and have been associated with 14 -37 % response rates. The most effective multidrug studies have included cisplatin, vinblastine and dacarbazine and have produced response rates of 40% with median duration of 9 months and a median survival of 12 months \(^9^0\).

3.4.2. Cytokine-based therapy

These therapies fall into the category of non-specific immunomodulation and involve mainly administration of IL-2 (Proleukin), INF\(\alpha\) and IFN\(\gamma\). IL-2 is a T cell growth factor that can activate endogenous tumor specific T cells and cause regression of metastatic cancer \(^9^2, 9^3\). Treatment with bolus high-dose IL-2 leads to various grades of toxicity ranging from chills and tremors to
capillary leak syndrome\textsuperscript{94} which may result in severe organ failure. However, administration of this cytokine has resulted in very low incidences of treatment related mortalities (<1\%)\textsuperscript{95}. The overall response rate of 270 melanoma patients enrolled in clinical trial at the National Cancer Institute and that were treated with IL-2 was 16\% with 6\% complete responses and median survival of 11.4 months\textsuperscript{96}.

Administration of IFN\(\alpha\) as monotherapy has lead to response rates of approximately 10\%\textsuperscript{97}. Several clinical studies have combined chemotherapy with IL-2 and IFN\(\alpha\) therapies. Combination therapy of IL-2, IFN\(\alpha\) and dacarbazine has produced response rates as high as 52\%\textsuperscript{98}. In another randomized phase III trial it was concluded that there was no significant difference in survival and response rates between dacarbazine and IFN\(\alpha\) (18\%) and dacarbazine, IFN\(\alpha\) and IL-2 (16\%)\textsuperscript{99}. Other cytokines such as IL-12, IL-15, IL-18 and granulocyte macrophage colony-stimulating factor have been tested as monotherapy or in combination with other agents but has so far resulted in low response rates\textsuperscript{100-104}.

3.4.3 Cancer vaccines
Active immunization of cancer patients with autologous cancer cells, proteins, peptides and various immunization vectors expressing tumor antigens have been attempted\textsuperscript{105}. However, even though high numbers of circulating anti-tumor CD8\(^+\) cells were generated, cancer progression still occurred\textsuperscript{106}. Vaccination of melanoma patients have shown modest results with only a minority of patients (5-10\%) achieving objective clinical responses\textsuperscript{107}. This is probably due to low avidity of the tumor-specific cells and to the existence of endogenous inhibitory cells within the tumor area\textsuperscript{106}.

3.4.4 Antibody-mediated therapy
Antibody-mediated blockade of the cell surface inhibitory molecule, cytotoxic T-lymphocyte associated 4 (CTLA-4) has resulted in objective clinical responses in 10-20\% of patients with melanoma or renal cancer\textsuperscript{108}. In a recent phase III study with the anti-CTLA-4 monoclonal antibody Ipilimumab, treating 676 stage III or stage IV melanoma patients, it was concluded that Ipilimumab with or without gp100 peptide vaccine, as compared to peptide vaccine alone, improved overall survival. The median survival was 10 months among patients receiving ipilimumab, as compared with 6.4 months in patients receiving gp100 alone. The best overall response rate was observed for ipilimumab alone (11\%). In the cohort of 676 patients, 3 patients showed complete response\textsuperscript{109}. Undergoing investigations are also evaluating the effects of anti-TGF and anti-programmed death 1 (anti-PD-1) antibodies.
3.4.5 Adoptive cell transfer therapy

The first attempts to treat established rodent tumors with adoptive cell therapy (ACT) was performed in 1960s when rats with sarcomas were treated with large numbers of lymphocytes obtained from immunized syngeneic animals. A decade later, studies revealed that transfer of immune lymphocytes together with chemotherapy could effectively treat virus-induced lymphomas. In the 1970s, techniques for in vitro culturing and expansion of tumor specific lymphocytes were established and replaced the need for fresh cells from immunized hosts. The first isolation and characterization of TILs from non-immunized melanoma or sarcoma-bearing host was done in 1986. These TILs were expanded in vitro using purified IL-2 and were used successfully to treat established lung and liver metastasis. More recently, effective ACT of large, vascularized and metastasized tumors with lymphocytes from TCR transgenic mice expressing anti-gp100 tumor antigen TCRs has defined important host factors and cell properties associated with successful therapy. One important discovery was the need for prior lymphodepletion of host before ACT for elimination of Treg and other suppressor cells as well as the elimination of cells that compete with the transferred cells for homeostatic cytokines IL-7 and IL-15. Better results were obtained with increased intensity of lymphodepletion to the level of myeloablative irradiation (TBI) that require hematopoietic stem cell (HSC) transplantation, before ACT. TBI together with HSC transplantation and transfer of large numbers of TILs greatly improved the outcome of the therapy.

Isolation of TILs from melanoma patients was initially done in 1987, these cells could be cultured in IL-2 containing medium and recognized autologous melanoma cells in a HLA-restricted manner. Improvements in culturing and expansion techniques enabled the generation of $10^{11}$ TILs with melanoma specificity in 81% of 36 consecutive patients. The first attempts of ACT in 86 patients treated with autologous TILs and high dose IL-2 resulted in 34% objective response rates. Persistence of transferred TILs in these initial studies were short and PCR assays revealed that 1 week after ACT, only 0.01% of the cells in circulation were transferred cells.

In recent trials at the Surgery Branch, National Cancer Institute, TIL cultures are initiated from multiple resections of fresh melanoma biopsies and are cultured in IL-2 supplemented medium in vitro. Cultured TILs are rapidly expanded using an agonistic anti-CD3 antibody (OKT3) and IL-2. Approximately $5 \times 10^{10}$ cells are infused following a non-myeloablative, lymphodepleting preparative regimen consisting of 60 mg/kg cyclophosphamide for 2 days followed by 5 days of fludarabine at 25 mg/m². In evaluation of increased lymphodepletion, fifty patients also received TBI in addition to lymphodepletion, 25 patients received 2 Gy and 25 received 12 Gy irradiation.
High dose IL-2 (720,000 IU/kg) was administered every 8 hours for 2-3 days. Objective response was seen in 49% patients who did not receive TBI\textsuperscript{122}. In the cohorts that received irradiation 13 of 25 (52\%) patients who received 2 Gy and 18 of 25 (72\%) of patients who received 12 Gy showed objective responses\textsuperscript{117}. Increased survival with increased lymphodepletion could be proven as the patients receiving ACT with non-myeloablative chemotherapy or TBI had a 3-year survival rate of 25\% and 42\% respectively, as compared with 14\% for the group without lymphodepletion\textsuperscript{117}. Important findings in these trials were that cell administrations did not add to toxicity and the observed toxicities were due to administration of IL-2. Furthermore, in responding patients, transferred cells persisted and expanded in vivo for as long as 12 months after infusion and persisting cells had longer telomeres which correlated with cancer regression\textsuperscript{123}. Transferred TILs were of late effector phenotype \textit{i.e.} CD27\textsuperscript{−}, CD28\textsuperscript{−}, CD45RA\textsuperscript{−}, CD62L\textsuperscript{−} and CCR7\textsuperscript{−}. However, two months after transfer the cells that were detected in circulation had re-expressed many of the differentiation markers (CD27\textsuperscript{+}, CD28\textsuperscript{+}) \textit{in vivo} and had a less differentiated phenotype\textsuperscript{124}. 
4. Adoptive cell therapy for treatment of malignant melanoma at Uppsala University and University Hospital

4.1 Methods

Encouraged by the high rates of cancer regression seen in the trials at the National Cancer Institute, we initiated a clinical trial in 2005 where we attempted to isolate TILs for treatment of 30 melanoma patients.

4.1.1 Preparation of TIL cultures from tumor tissue

There are several protocols for isolation of TILs from tumor biopsies\textsuperscript{119,125,126} and they all involve either mechanical fragmentation or chemical decomposition of fresh melanoma biopsies. Processed biopsies are cultured in IL-2 supplemented complete medium (CM) which stimulates TIL migration out of tumor tissue for subsequent proliferation in culture plates.

TIL preparation and culturing were performed according to Good Manufacturing Practice conditions at the Clinical Cell Therapy Laboratory, Clinical Immunology Division, Rudbeck Laboratory. Tumor biopsies were obtained either through surgery or ultrasound-guided needle biopsy and were immediately placed in CM after resection. In surgically removed specimens the tumor was dissected from surrounding normal tissue and small pieces were cut (measuring 3x3x3 mm). To minimize blood contamination, every piece was gently washed in excess of CM before added to a 12-well tissue culture plate with 1 ml of CM supplemented with 6000 IU IL-2. In the case of needle biopsy the material was suspended in CM and whole biopsies were cut into pieces (measuring 2x2x2 mm) before being added to IL-2 supplemented CM as above. The plates were incubated and each well was inspected by microscopy for lymphocyte growth. Cultures were fed with fresh CM (supplemented with 6000 IU/ml IL-2) on basis of lymphocyte proliferation and medium exhaustion until end of experiment. Highly proliferating cultures were also expanded in larger culturing containers. In total 728 TIL cultures were initiated (12 to 33 cultures per patient).
4.1.2 Analysis of TILs

Tetramer analysis

TILs were analyzed for TCR-peptide-specificity through tetramer analysis. The HLA-A*0201/Mart1, Tyrosinase, gp100 and Her-2/Neu tetramers were used for analysis of cells from HLA-A*0201+ patients. The majority of positive TIL cultures were specific for MART-1 (53%, 39/73) and gp100 (32%, 23/73) tetramers and only sporadic reactive cultures were observed with the two additional tetramers.

IFNγ analysis

A total of 318 cultures originating from all 30 patients were analyzed for IFNγ production upon stimulation with one or several HLA-semi matched melanoma cell lines. As negative control we used one or several HLA unmatched melanoma cell lines. We used a protein transport inhibitor to block secretion and analyzed for intracellular production of IFNγ.

To analyze the expression of some known general solid tumor, cancer-testis and melanoma differentiation antigens we performed RT-PCR for a total of 21 genes. MART-1/MelanA, gp100 and tyrosinase, all extensively exploited in melanoma immunotherapy, showed intense PCR amplicons in all melanoma cell lines (Table III in paper I).

Quantitative Real-time PCR (qRT-PCR)

Quantitative RT-PCR was performed to determine the gene expression levels of pro/anti-apoptotic markers and inhibitory molecules of lymphocytes prior to and after rapid expansion. The panel of analyzed genes (Table 1, paper III) consisted of markers for: immunoregulation (IL-10, FOXP3, TGF-β), pro-apoptosis (P73, FasL), anti-apoptosis (BCL-2) and β-actin as housekeeping gene.

Cylex Immuknow® immune function test

Immuknow is a test for monitoring of immune responses in transplanted patients. This test combines cell stimulation, cell selection and quantification of Adenosine triphosphate (ATP) as an indicator of cellular activation. We measured the intracellular activity of lymphocytes for each step of the expansion protocol by stimulation with Phytohaemagglutinin (PHA). We made a positive selection of CD4+ cells, and lysed the cells to measure intracellular ATP concentrations.
4.1.3 Rapid expansion of TILs

By initially starting 24 wells containing tumor fragments and 2ml CM, about 5-6 x10^7 TILs were generated on average in 28 days for the first 24 patients. The last four treated patients received young TILs. Generation of young TILs involved minimal culture time in separate wells of a 24-well plate, with multiple individual tumor fragments. After the expansion and confluence, TILs from individual wells were pooled and rapidly expanded. This process took on average 21 days (Figure 6).

Rapid expansions of TILs were performed using the Rapid Expansion Protocol. TILs were cultured in standing T flasks with 200-fold excess of irradiated allogeneic blood mononuclear cells from several healthy donors as feeder cells. Cells were cultured in CM with 5% human AB serum, 30 ng/ml agonistic anti-CD3 antibody and 600 IU/ml IL-2. The cells were transferred to larger T flask or gas-permeable bags in order to maintain a concentration of 1.5 – 3 x 10^6 cells/ml. An average of 300 fold expansion was observed. Final numbers of administered TILs varied based on growth rates of cells.

4.1.4 Adoptive cell therapy of melanoma patients

One week prior to transfer of autologous cells, patients received nonmyeloablative lymphodepleting preconditioning regimen consisting of 2 days of cyclophosphamide (60 mg/kg) followed by 5 days of fludarabine (25mg/m^2). Low dose (2.4 x 10^6 units/m^2) IL-2 therapy was initiated on the day after the final dose of chemotherapy and was followed by immediate infusion of TILs. A mean of 7 x 10^9 TILs were infused (range, 0.5 to 30 x 10^9). Monitoring of response was through CT scans at 4 weeks following cell infusion and thereafter when appropriate on an individual basis. Patient responses were categorized by two experienced radiologist in accordance with the latest RECIST criteria.
Figure 6. Isolation of TILs for adoptive cell therapy. Minimally cultured cells are obtained after plating multiple tumor biopsies in 6000 IU/ml IL-2. After migration and expansion, the cells become confluent and are pooled before rapid expansion. After lymphodepletion of host, large numbers of expanded cells \((10^9 - 10^{10})\) are used for adoptive therapy.
4.2 Results and Discussion

Paper I

In this study we sought establish a protocol for the assessment of melanoma TIL culture and reactivity. To test TIL reactivity in a robust, reliable and simple manner we wanted to find whether HLA matched melanoma cell lines could be used for this purpose. Several melanoma cell lines were used to analyze TIL for intracellular IFNγ production. In the case of HLA A2+ patients, we also performed stimulation with peptide-pulsed cells and tetramer analysis. The patients had stage III – IV melanoma progression and were refractory to conventional therapy. Tumor tissue was obtained from subcutaneous, lymph node and internal organ metastases and was removed by either ultra-sound or CT guided needle biopsy or through surgery. Our results confirm previous studies showing a highly reproducible expansion of TIL populations in the majority of the patients. We initiated 728 individual TIL cultures in total and could observe lymphocyte growth in 444 (60.9%) of the cultures of which 318 (46%) reached cell numbers above 100×10⁶. We processed 15 needle biopsies and 18 surgically removed tissue specimens and could observe TIL growth in 45% and 73% of cultured tissues respectively. 318 cultures were analyzed for IFNγ producing cells upon stimulation with melanoma cell lines. Of these 29% displayed IFNγ activity while IFNγ production was never detected after stimulation with HLA un-matched melanoma cell lines. We could not observe any significant difference between TILs originating from needle biopsies or surgical material with regard to reactivity. Of the tetramer positive TIL cultures, 53% (39/73) were specific for MART-1 and 31% (23/73) were positive for gp100. The major conclusion was that reactive cells can be found in a majority of patients and can be screened for specificity using melanoma cell lines. Furthermore, the method is highly relevant for the many patients from whom no primary tumor cells can be established.

Paper II

We treated 28 patients with progressive stage IV malignant melanoma with an average age of 51 years (range 17 – 73) at Uppsala University Hospital between June 2005 and January 2010. All patients were refractory to standard treatments including surgery and several lines of chemotherapy and radiotherapy. The majority of the patients (25 of 28) had a good performance status (WHO 0-2) and three patients had a WHO status of 3. We observed an objective response rate of 18% including one complete remission and four partial responses. Tumor regression was demonstrated in additionally six patients. Average hospitalization after treatment was 18 days during which 14 of 28 patients had febrile neutropenia with neutrophil count <10⁹ for 5
days on average. During the in-patient period, 43% (12/28) of the patients required transfusion of platelets and 61% (17/28) transfusions of erythrocytes. One important finding in this study was the successful production and expansion of TILs obtained from ultrasound-guided needle biopsies (15 of 28 patients). In this group of patients we observed 1 CR, 3 PR and 5 SD, making this less strenuous method of obtaining biopsies, superior to surgery.

This was the first study in cancer patients utilizing autologous T cell transfer in combination with non-myeloablative but lymphodepleting chemotherapy and support with low dose IL-2. Other similar trials have shown higher rates of objective response. One explanation for this is more rigorous selection of patients and TIL populations used in therapy. We could not justify discarding of TILs and denial to treat patients that had undergone biopsies, no matter how low the growth rate or the final numbers of the expanded TILs were. Other discrepancies that may have had an effect on the outcome were the lack of total body irradiation, weaker anti-tumor effects due to administration of low dose IL-2 and lower numbers of infused TILs. There have to our knowledge so far not been any studies to examine the correlation between the number of transferred TILs and the outcome of therapy. In the animal model it is clear however that the quality of the T cells is more important than the quantity as relatively small numbers of highly specific and avid cells result in considerable tumor regression. In conclusion, we observed objective responses (18%) including one sustained complete response when treating melanoma patients with lower numbers of TILs, expanded in low dose IL-2 (600 IU/ml) with accompanying low dose subcutaneous IL-2 therapy.

Paper III

Mass expansion together with cryopreservation of cells is important and inevitable steps in most cellular therapies. Management of cells prior to and after large-scale expansion for ACT usually involves two freezing and thawing steps. The first step is performed after isolation and the second after mass expansion of the cells. Induction of massive and rapid clonal expansion together with cryopreservation can be a rather harsh treatment of tissue with the potential to induce considerable alterations in cell functions, phenotype and viability. This study was designed to assess the effects of cryopreservation when combined with the widely used protocol for rapid expansion of lymphocytes. We demonstrated that immediately after thawing, the lymphocytes show none to very low activity after mitogenic stimulation. The cells were however able to regain normal levels of intracellular activity when allowed to recover. Furthermore, cryopreservation during different time points in the rapid expansion protocol did not affect cell viability or the expression of markers for inhibition and apoptosis. These markers were however affected by rapid expansion. In conclusion, rapid expansion of lympho-
cytes could lead to loss of antigen specificity and apoptosis that was reduced after short time recovery. This could be important for future refinements of adoptive T cell therapies.

Paper IV

In this study we set out to evaluate the expansion of TILs in the WAVE Bioreactor system with perfusion. The bioreactor, in contrast to traditional culturing methods, allows for an automated provision of fresh and removal of spent media while minimizing culture volumes. This could lead to a better quality while allowing higher scales of cell expansions. We concluded that the bioreactor could be used for large-scale expansion of TILs and allows for higher cell densities and absolute cell numbers as compared to static culture conditions. The two expansion methods had no influence on the Phenotype of the TILs. Bioreactor expansion of TILs proved to be a reliable and labor saving alternative to traditional expansion methods.
Future perspectives

What is most encouraging about adoptive cell therapy, other than the current results, is that contrary to many conventional therapies, ACT holds much room for improvement and enhancements. The introduction of genes coding antigen receptors and molecules that provide the lymphocytes with enhanced properties is today being extensively studied. These genes are isolated from highly avid T cells and are introduced to T cells through retroviral or lentiviral vectors in order to re-direct T cell specificity. These high-affinity T cells can be isolated from rare reactive clones that are too few in number and under suppression in vivo. Administration of antibodies that that block inhibitory signals such as anti-CTLA-4 or PD-1 on reactive T cells together with ACT is a promising approach. The anti-tumoral effects of these antibodies in combination with ACT are yet to be elucidated. It has been proven that increased persistence of transferred cells can be correlated with increased efficacy of the therapy. The in vivo persistence can be further improved through administration of alternative cytokines such as IL-15 and IL-21 or through activation of APCs with Toll-like receptors. The management and culturing conditions can also be improved in order to generate more vigorous T cells that are less differentiated. One important improvement is to make the therapy more accessible and less cost and labor-intensive. The development of closed system technologies and bioreactors could circumvent the need for extensive regulatory aspects and the need for expensive clean rooms. ACT must in the future be viewed as a service rather than a personal and patient-specific drug.
Vårt immunförsvar kan delas upp i naivt och specifikt (adaptivt) försvar varav det senare är det framträdande i försvaret mot cancer. Det specifika försvaret består av T och B lymfocyter. Cirkulerande lymfocyter exponeras för främmande ämnen s.k. antigener genom antigenpresenterande celler vilka även bidrar med aktiveringssignaler som får lymfocyterna att expander klonalt samt att söka sig till källan av antigenen. Ösraken till förekomsten av främmande antigener i kroppen kan vara invadering av kroppsfrämmande patogener eller onormalt uttryck av kroppsegna proteiner som oftast sker hos cancerceller.

T-celler har till uppgift att förgöra källan av antigenen och kan delas upp i cytotoxiska T celler, hjälpar T celler och minnes T celler. Immunförsvarets främsta vapen för bekämpning av cancer är cytotoxiska T celler som förstör cancerceller genom cell-cell kontakt eller genom produktion av effektormolekyler.

Malignt melanom drabbar årligen ca 2000 svenskar vilket gör den till en av de vanligare cancersjukdomarna i Sverige. Dessutom leder den till 400 relaterade dödsfall. Till skillnad från många andra cancerformer anses malignt melanom vara immunogen, dvs. innehålla förmågan att attrohera kroppens egna T celler till att angripa tumören. Man har kunnat påvisa några få fall av spontan cancerregression av primär tumör.

Immunterapi av cancer har under senare år visat lovande resultat för behandling av flera immunogena cancerformer såsom: malignt melanom, njurcancer och tjocktarmscancer. En form av immunterapi är adoptiv cellterapi vilket innebär isolering av immunceller från blod eller tumörvävnad från cancerpatienter. Dessa celler karaktäriseras och expanderas i antal utanför kroppen och ges tillbaka till patienten. Tack vare att malignt melanom har immunogena egenskaper är det möjligt att isolera betydliga mängder T celler från tumörsbiopsier tagna från melanompatienter. Vi har, i vår första studie, visat att vi kan isolera tumörinfiltrerande lymfocyter (TIL) från 444 av 728 (60 %) biopsier tagna från patienter. Vi har vidare föreslagit metoder för analys av dessa TIL genom stimulering med melanomcellinjer. En sådan analys kan vara värdefull vid screening av olika populationer i jakten på de mest tumörreaktiva cellerna. Tumörspecifika celler kan massexpanderas i antal genom tillsats av olika tillväxtfaktorer. Expanderade celler ges sedan...
tillbaka till lymfopen patient och dessa T celler kan förstöra stora mängder av tumörceller och, i enstaka fall, bota patienter. Man inducerar lymfopeni genom kemoterapi för att skapa utrymme för nyinkomna TIL och undvika konkurrens från existerande T celler. I vår andra studie behandlade vi 28 melanompatienter med avancerad sjukdom som hade progredierat på etablerade behandlingar. Till skillnad från tidigare dylika studier behandlade vi patienter med lägre antal celler och efterbehandlade med lägre dos interleukin 2 vilken är en cytokin som främjar T-cellstillväxt i och utanför kroppen. Den låga IL-2 dosen tolererades, som väntat, betydligt bättre än vad som rapporterats då denna interleukin används i höga doser. Enligt bestämda kriterier för tumörminskning, svarade 18% på behandlingen. En patient svarade med långvarig komplett respons och fyra med partiell respons. Minskning av tumörmassan noterades för 6 ytterligare patienter (21%) utan att kriterierna för partiell respons uppfylldes. Resultaten av studien är lovande men modifieringar av behandlingsschemat behövs för att höja den antitumorala effekten.


Dessa studier har visat att administrering av tumörspecifika T-celler riktade mot ett lämpligt mål kan mediera regression av metastaserande cancer hos människor och ge vägledande principer samt uppmuntran till en vidareutveckling av immunterapi för behandling av patienter med cancer.
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5. References


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