Tumor-Induced Immune Dysfunction: Mechanism and Therapeutic Strategies

Mikael Hanson
Doctorial Thesis
Tumor-Induced immune Dysfunction: Mechanism and Therapeutic Strategies
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To my family
Abstract

Cancer is one of the major causes of premature death in humans. Although standard treatments, such as surgery and chemotherapy, are successful in many cases, there are instances when their utility and efficacy are limited. Immunotherapy against cancer has recently been developed as an example of a new generation of “targeted” therapy. However, the immunosuppressive milieu associated with tumors is an obstacle that needs to be overcome to improve the response rates of immunotherapeutic approaches.

Several biological processes are involved in the induction and resolution of an immune response and these need to be in perfect equilibrium to allow optimal functioning of the immune system. However, this balance is skewed in cancer patients creating a state of chronic inflammation that in turn results in a suppression of the immune system leading to tumor-induced immune dysfunction. Several mechanisms have been suggested to contribute to this phenomenon, including mechanisms that induce oxidative stress in cancer patients.

The aim of this thesis is to elucidate the effects of oxidative stress on lymphocytes and define methods of reducing it. We have shown that oxidative stress affects competent anti-tumor cells, such as CD8\(^+\) T effector memory cells and CD56\(^{dim}\) NK cells, most as compared to other cells in the immune system. This may provide one explanation for the limited clinical response noted with active immunotherapy against cancer. To counteract oxidative stress, we have developed two approaches; firstly, we have been able to increase NK cell function in cancer patients by oral administration of the antioxidant vitamin E. Secondly, by transferring a gene encoding for the antioxidant enzyme catalase we were able to increase the antioxidant capacity in lymphocytes and improve their ability to resist oxidative stress.

The work performed within this thesis furthers the understanding of oxidative stress-induced suppression of lymphocytes. This study has also contributed to the development of approaches for reversing this suppression. Methods for reversing oxidative stress-induced immune dysfunction may potentially improve the clinical outcome of subsequent active immunotherapy regimens against cancer.
List of Publications


*Shared first authorship
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<th>Full Form</th>
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<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>8-OxoGua</td>
<td>8-oxo-2-deoxyguanosine</td>
</tr>
<tr>
<td>ACAD</td>
<td>Activated cell autonomous death</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>APM</td>
<td>Antigen presentation machinery</td>
</tr>
<tr>
<td>ARG1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl2-antagonist of cell death</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 Homologous Antagonist Killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma-2</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 Homology</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>Bik</td>
<td>Bcl-2-interacting killer</td>
</tr>
<tr>
<td>Bim</td>
<td>BCL-2-interacting mediator of cell death</td>
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<tr>
<td>Caspase</td>
<td>Cysteine-dependent aspartate-directed proteases</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CCR7</td>
<td>Chemokine receptor-7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD95L</td>
<td>CD95-ligand</td>
</tr>
<tr>
<td>cdk4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DIABL0</td>
<td>Direct inhibitor of apoptosis-binding protein with a low isoelectric point,</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GM-CFS</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Disulfide glutathione</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis proteins</td>
</tr>
<tr>
<td>ICAD</td>
<td>Inhibitor of caspase-activated Dnase</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric-oxide synthase</td>
</tr>
<tr>
<td>LMP</td>
<td>Low molecular mass polypeptide</td>
</tr>
<tr>
<td>MCA</td>
<td>Methylcholanthrene</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Major histocompatibility complex class I chain-related</td>
</tr>
<tr>
<td>NCR</td>
<td>Natural cytotoxicity receptors</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKG2A</td>
<td>Natural killer group 2A</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO2</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose)polymerase</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-associated antigens</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCM</td>
<td>Central memory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptors</td>
</tr>
<tr>
<td>TEM</td>
<td>Effector memory T cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumor growth factor beta</td>
</tr>
<tr>
<td>TH1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor-infiltrating lymphocytes</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor-necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Introduction

Despite the great progress made in research, cancer still continues to be one of the major causes of premature death in humans. In 2003, 48 867 new cases of cancer were recorded in Sweden, which is twice as many as in the year 1970\(^1\). Many cancer types have a much better treatment prospect today compared to 20 – 30 years ago, while other more aggressive cancers, such as pancreatic cancer, still have a very poor prognosis. Thus, there is a great need for new and innovative cancer therapies to treat these cancers.

**Current Therapies of Cancer** Radical surgery is presently one of the first line treatments of solid tumors. It is an effective, yet drastic, way to eliminate tumor tissue. Unfortunately, many tumors are inoperable due to their anatomical location or to the extent of dissemination to the surrounding tissue. In many cases, the surgeon needs to remove healthy tissue, including lymph nodes surrounding the tumor, giving rise to other complications. In the case of non-solid tumors, e.g. leukemia, surgery is not an option. The second most common approach of cancer therapy is exposing the tumor to radiation. Radiotherapy has been used since the late 19\(^{th}\) century and constitutes 30 % of all treatments. This therapy is often used in combination with surgery or chemotherapy. However, despite its potential benefit, radiation can cause serious damage to the adjacent nonmalignant tissue. As for surgery, the anatomical location and spread of the tumor may restrict the potential utility of radiation in cancer therapy. The third major arm of cancer treatment is chemotherapy using cytotoxic drugs that preferentially kill cancer cells. Although the treatment with these drugs in several malignancies is effective, the adverse effects are often severe. The side effects of certain chemotherapy regimens are severe enough to preclude their use in the treatment of the elderly or patients whose health is compromised. The limitations of existing treatment approaches underline the need for novel therapies. The field of potential cancer therapies is evolving and is now extended to involve many medical disciplines. For example, the introduction of hormone and receptor antagonists for the treatment of hormone-dependent cancers has improved the prognosis of advanced cancers. Tamoxifen, an antagonist of the estrogen receptor, has been successfully used in treating breast tumors that are dependent on the estrogen receptor. However, a significant number of patients develop recurrences that are resistant to tamoxifen treatment\(^2\).
The progress in chemo/radiotherapy and surgery together with other approaches like hormone antagonists have greatly improved the survival in many cancers, but there is still considerable room for improvement. One approach of treating cancers involves the use of immunological entities, also known as immunotherapy. Passive immunotherapy entails the administration of therapeutic antibodies whereas active immunotherapy, popularly known as cancer vaccines, relies on stimulating the body’s own immune defense for the eradication of the tumor cells. Presently, at least seven antibodies are licensed for the treatment of cancer; however, active immunotherapy approaches have not achieved the success that the scientific community had hoped for.

The limited success of active immunotherapy against cancer may be attributed to several factors (described in detail in section 2). In the progression of the disease, cancer cells evolve strategies to avoid an immune response through loss of components responsible for the presentation of cancer-specific antigens and/or loss or gain of cellular functions that renders the cancer cell resistant to cell death. These mechanisms are intrinsic to the cancer cells and do not have an immediate effect on the tumor microenvironment. Concurrently, cancer cells are in constant interaction with their surroundings. The cancer cell progressively mutates, thereby acquiring traits that enable the cell to utilize a variety of normal physiological and biological processes to its advantage. These processes have been named tumor-induced immune dysfunction and can be summarized as the tumors ability to manipulate the physiological functions of the tumor stroma (normal tissue surrounding the tumor), the immune system, and the healing process. The focus of this thesis is to investigate mechanisms and possible therapeutic strategies to reverse tumor-induced immune dysfunction. An awareness of the basics of inflammation and resolution of inflammation is vital to understanding this thesis, as these physiological events also play a critical role in tumor-induced immune dysfunction.

1. The Start and Stop of Inflammation

The inflammatory process has evolved to prevent an infection or a “xenobiotic insult”. Inflammation is induced in response to a traumatic tissue damage or an infection and is a complex process involving cells and soluble factors. The regulation of inflammation is tightly governed by pro- and anti-inflammatory signals and, in normal condition, leads to the eradication of the infectious agents
and to tissue repair\(^7\). For reasons of simplicity, this section describes the typical inflammation caused by non-infectious tissue damage (mild trauma) with respect to the chronic inflammation seen in tumor tissues.

## 1.1. Onset of Inflammation

In response to mild trauma, tissue resident leucocytes (e.g. mast cells and macrophages) perceive that a potential infectious agent may have penetrated the skin and infected the tissue. To counteract this possible infection, mast cells, activated by neuropeptides released by neurons in response to pain, secrete several effector molecules, e.g. histamine, pro-inflammatory leukotrienes, prostaglandin E\(_2\) (PGE\(_2\)), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and chemokines\(^8\).

Histamine, leukotrienes, and PGE\(_2\) cause vasodilatation (responsible for the heat and redness seen in inflammation) and extravasation of fluid (responsible for the swelling seen in inflammation)\(^8\). The trauma also causes cell disruption, thus releasing constitutively expressed intracellular proteins, such as heat-shock proteins\(^9\), the transcription factor high mobility group box 1\(^10\) (HMGB1) and mitochondrial peptides resembling prokaryotic components\(^11\). When detected by tissue-resident macrophages, these proteins trigger activation and the release of TNF-\(\alpha\) and chemokines by the macrophage\(^7\).

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![Fig 1 - Activation of the inflammatory response.](image)

In response to mild trauma mast cells and macrophages become activated and secrete factors that attract cells from the innate and adoptive immune system to the site of inflammation. Hsps: heat-shock proteins, HMGB1: high mobility group box 1, TNF-\(\alpha\): tumor necrosis factor-\(\alpha\), PGE\(_2\): prostaglandin E\(_2\). Adapted from Nathan\(^7\).
The pro-inflammatory molecules also activate the endothelium of blood vessels to express various cell adhesion molecules, such as E- and P-selectin, needed for leukocyte rolling and transmigration from the peripheral blood into the damaged tissue\textsuperscript{12}. Once adhered to the endothelial wall of the activated blood vessel, chemoattractants direct the leukocyte to the site of inflammation. The first cell type to migrate to the inflamed tissue is the neutrophil\textsuperscript{7}. The neutrophil is a cell with a short lifespan and phagocytic capacity. It is the most abundant cell in the blood and upon tissue damage will accumulate at the site of inflammation within hours of insult\textsuperscript{12} (Fig 1). At the site of tissue damage, TNF-\(\alpha\) activated neutrophils secrete various enzymes, such as myeloperoxidase, elastase, matrix metalloproteases, and cathepsins that decompose extracellular matrix components. Furthermore, the neutrophils secrete reactive oxygen species (ROS) through an oxidative burst\textsuperscript{7,12,13}. The events described above lead to further neutrophil infiltration, thus potentiating the inflammatory process.

Peripheral blood monocytes are next to enter the site of inflammation, guided by chemoattractants produced during the initial inflammatory response. Once activated in the damaged tissue, monocytes differentiate into macrophages or immature dendritic cells (DCs)\textsuperscript{14}. The macrophages now act as the main source of growth factors and cytokines that modulate both inflammation and resolution of inflammation\textsuperscript{15} (see section 1.2 for details). Macrophage- and monocyte-derived TNF-\(\alpha\) and chemoattractants recruit and activate more neutrophils. The combination of neutrophil-derived products, e.g. anti-bacterial peptides and mast cell-derived PGE\(_2\), attracts lymphocytes\textsuperscript{16} and leukotrienes recruit DCs to the site of inflammation\textsuperscript{17}, thus ensuring a response by the adaptive immune system. Cytokines produced by lymphocytes together with microbial-like products, e.g. formyl-peptides released from necrotic cells, trigger macrophages to release antimicrobial molecules, e.g. ROS and reactive nitrogen species (RNS)\textsuperscript{7}.

In summary, the onset of an inflammation is a cascade of events initiated by cells and soluble factors. It is an immediate response that ensures a quick and efficient removal of infectious agents. As a simplification, one may consider the immediate inflammatory response to have two purposes 1) to ensure an infiltration of immune cells from peripheral blood into the damaged tissue and 2) to combat the infectious agent at the site of inflammation. To facilitate cellular infiltration a cascade of cytokines and chemokines are released through different means and to execute an immediate removal of infectious agents, macrophages and neutrophils
secrete various anti-microbial molecules, e.g. ROS and anti-bacterial peptides. In addition, the initiation of inflammation also triggers an essential process, i.e. resolution of inflammation and tissue repair.

1.2. Resolution of Inflammation

Normally, inflammation caused by mild trauma quickly declines and the tissue heals. To this end, the switch from an anti-bacterial, tissue-damaging mode to a tissue repair mode needs to be made at the closing stages of inflammation. The onset of macrophage infiltration into the inflamed tissue coincides to the decline in numbers of neutrophils at the site of inflammation\(^\text{15}\). As long as there are pro-inflammatory components, e.g. HMGB1 and formyl-peptides, macrophages will produce chemokines to attract peripheral blood neutrophils\(^\text{7}\). The transformation from inflammation to tissue repair initiates when the amount of pro-inflammatory components decreases and when macrophages, at a late stage of inflammation, start to produce secretory leukocyte protease inhibitor (SLPI), a serin protease inhibitor with anti-inflammatory\(^\text{18}\) and tissue repair-promoting effects\(^\text{19}\). The release of SLPI by macrophages 1) suppresses neutrophil infiltration into the inflammatory site by inhibiting the secretion of chemoattractants, 2) inhibits ROS production by neutrophils\(^\text{20}\) and 3) prevents tumor growth factor-β (TGF-β) break down\(^\text{19}\). The remaining neutrophils undergo apoptosis and macrophages start to ingest apoptotic neutrophils\(^\text{7}\). The phagocytosis of apoptotic bodies derived from neutrophils initiates macrophages to produce TGF-β\(^\text{7}\). In turn, the production of TGF-β starts the process of tissue repair.

Concurrent to the activation of the innate immune response, cells of the adaptive immune response enter the site of inflammation and mount a humoral and/or cellular adaptive immune response. For most infections, the cellular component of the adaptive immune response needs to be reduced to minimize tissue damage and autoimmunity once the infectious agent is cleared. This is mediated by several physiological processes such as 1) regulation of T cells and natural killer (NK) cells through cell death, 2) deprivation of essential nutrients necessary for lymphocyte function at the site of inflammation, and 3) production of immune suppressive cytokines.
Regulation of Inflammation through Cell Death of T Cells and NK Cells

It has been shown that induction of cell death in T cells and NK cells regulates the lymphocyte immune response. There are two distinct mechanisms in which induction of cell death plays a key role, i.e. activation-induced cell death\(^ {21} \) (AICD) and activated cell autonomous death\(^ {22} \) (ACAD). Upon encounter and recognition of antigens presented on DCs, T cells clonally expand and home to the site of inflammation\(^ {23} \). To counteract possible autoimmune reactions, T cells that are activated without proper co-stimulation are eliminated through AICD\(^ {23} \). Over time, the majority of the expanded T cells gain traits that make them susceptible to cell death, while some T cells differentiate into a memory phenotype with an increased resistance to AICD\(^ {24} \). The induction of cell death in activated T cells acts as a safety mechanism assuring that the effector phase of the adaptive immune response is transient and declines when the infectious agent has been cleared. It has been shown that the increased susceptibility of activated T cells to cell death is due to increased expression of cluster of differentiation 95 (CD95), thus sensitizing the cells to CD95-mediated cell death by autocrine or paracrine CD95-ligand (CD95L) expression\(^ {25} \). Other studies have also indicated a role for TNF-\( \alpha \)\(^ {26} \) and tumor-necrosis factor-related apoptosis-inducing ligand\(^ {27} \) (TRAIL) in the induction of AICD. In addition, it has recently been shown that NK cells also commit to AICD as interleukin-2 (IL-2) stimulated NK cells express and release CD95L upon natural cytotoxicity receptors (NCR) engagement with target cells, thus mediating suicide of NK cells expressing CD95\(^ {28} \).

The absence of survival signals also contributes to ACAD in T cells\(^ {22} \). ACAD is regulated by the balance between intracellular anti- and pro-apoptotic proteins (mechanisms of cell death are explained in detail in section 3). It has been shown that during expansion, T cells downregulate the expression of the anti-apoptotic protein B-cell lymphoma (Bcl)-X\(_L\)\(^ {29} \) and upregulate the expression of the pro-apoptotic proteins Bcl-2-interacting mediator of cell death (Bim) and p53-upregulated modulator of apoptosis (PUMA)\(^ {30-32} \). This shift in the balance renders the T cells sensitive to cell death stimuli.

Deprivation of Essential Nutrients \( \text{L} \)-arginine is an essential amino acid needed for synthesis of proteins. \( \text{L} \)-arginine is mainly catabolized in vivo by arginase 1 (ARG1) and inducible nitric-oxide synthase (iNOS) to produce urea and \( \text{L} \)-ornithine, and nitric oxide (NO) and \( \text{L} \)-citrulline, respectively\(^ {33,34} \). It has been proposed that in higher organisms metabolism of \( \text{L} \)-arginine regulates unwanted T
cell expansion\textsuperscript{35}. Recently, Rodriguez et al. showed that \( \text{L}\)-arginine starvation impairs the expression of cyclin D3 and cyclin-dependent kinase 4 (cdk4) in T cells, thus inhibiting downstream signaling leading to G0-G1 cell cycle arrest\textsuperscript{36}. In parallel, catabolism of tryptophan has been proposed to downregulate the T cell response at the maternal-fetal interface. It has been shown that expression of indoleamine 2,3-dioxygenase (IDO), which catalyzes \( \text{L}\)-tryptophan conversion into kynurenine, is important for the establishment of the immune privilege site and that starvation of \( \text{L}\)-tryptophan leads to cell cycle arrest\textsuperscript{37}.

**Suppressive Cytokines** In the late stages of the inflammatory process the switch from inflammation to resolution and tissue repair is orchestrated by immune suppressive (or pro-tissue repair) cytokines. TGF-\( \beta \) expressed in the later phase of inflammation inhibits activation, effector function, proliferation and differentiation of T cells\textsuperscript{38,39}. TGF-\( \beta \) is an important regulator of the immune system as deficiency in TGF-\( \beta \) results in lethal autoimmunity in mice\textsuperscript{40,41}. TGF-\( \beta \) has also been shown to inhibit NK cells by inducing downregulation of the activating NK cell receptors Nkp30 and natural killer group 2D (NKG2D)\textsuperscript{42} and enhance the expression of the inhibitory receptor CD94/NKG2A in T cells\textsuperscript{43}. TGF-\( \beta \) also drives the expansion of regulatory T cells (T\textsubscript{reg}), a T cell subset responsible for controlling the immune response\textsuperscript{44-47}. In addition, IL-10 reduces the immune response by suppressing the production of pro-inflammatory cytokines, such as interferon-\( \gamma \) (IFN-\( \gamma \)) and IL-2, and impairs DC function, thus limiting priming of T cells\textsuperscript{48,49}. Furthermore, vascular endothelial growth factor (VEGF) has also been shown to delimit DCs ability to present antigens to T cells\textsuperscript{50}.

<table>
<thead>
<tr>
<th>Table I – Key molecules involved in inflammation and resolution of inflammation</th>
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<tbody>
<tr>
<td><strong>Induction of Inflammation</strong></td>
</tr>
<tr>
<td><strong>Inflammation initiator molecules</strong></td>
</tr>
<tr>
<td>Examples: Formyl peptides, bacterial products, HMGB1, neuropeptides, Hsp</td>
</tr>
<tr>
<td><strong>Inflammatory chemoattractants/cytokines</strong></td>
</tr>
<tr>
<td>Examples: TNF-( \alpha ), TGF-( \beta ), PGE( _2 ), Histamine, leukotrienes</td>
</tr>
<tr>
<td><strong>Toxic molecules</strong></td>
</tr>
</tbody>
</table>

In conclusion, there are factors that induce the inflammatory process and other factors that lead to the resolution of inflammation (see Table I for summary). These factors are programmed to be produced following a particular time course.
Figure 2 provides a simplified summary of the different factors involved in inflammation and resolution of inflammation and relates them to the time course of the various processes. Many of these factors have been shown to be implicated in tumor-induced immune dysfunction.

**Fig 2 - Induction and resolution of inflammation leading to tissue repair.**

Upon insult, tissue-resident mast cells and macrophages become activated by bacterial/viral-derived products, e.g. LPS and fMLP, or by products produced after tissue damage, e.g. neuro-peptides, HMGB1, and formyl-peptides derived from mitochondria of damaged cells. The activation facilitates the induction of inflammation during which the first wave of inflammatory cells entering the site of inflammation are neutrophils. Thereafter, monocytes enter the site and differentiate into macrophages. Neutrophils and macrophages facilitate antibiotic activity through secretion of toxic molecules, such as ROS. The next wave of immune cells to infiltrate the site of inflammation is T cells previously primed in secondary lymph nodes by DC migrating from the inflamed tissue. T cells recognize cells that express the antigen that they were primed against, e.g. viral proteins, and the kill target cells. When the signals from the insult have diminished, e.g. the infectious agent is cleared, the inflammatory response is converted to a pro-resolution state. For example, macrophages switch from pro- to anti-inflammatory, thus secreting immune suppressive cytokines, such as TGF-β. In parallel, lymphocyte activity is downregulated by induction of cell death and deprival of essential nutrients. The above described processes aid the subsequent tissue repair, e.g. angiogenesis, leading to healing of the damaged tissue.
2. Tumor Immunology

In the 1960’s the first experiments testing the immunogenicity of tumors were conducted. Mice immunized with attenuated, syngeneic tumors, were protected from a subsequent challenge with live tumor\textsuperscript{51,52}. This protection was tumor-specific as challenge with a different tumor, even with one induced by the same carcinogen, did not protect the mice from developing tumors. In contrast, tumors induced by virus, such as papilloma virus, share the same antigens and mice are protected from a subsequent challenge using a different tumor induced by the same virus\textsuperscript{53}. These observations formed the basis of the idea that a tumor-specific adaptive immune responses against non-virally-induced tumors could occur.

Several tumor-associated antigens (TAA) have been described\textsuperscript{54-56}. These TAAs can be categorized into 1) differentiation antigens, i.e. antigens that are expressed during some stage of the differentiation by normal cells in the same lineage, e.g. the melanoma antigen MART-1, 2) mutated antigens, i.e. antigens derived from mutated proteins, e.g. mutated p53, 3) overexpressed antigens, i.e. antigens that are expressed at low levels in normal cells, but are overexpressed in tumor cells, e.g. Her-2/neu, 4) cancer-testis antigens, i.e. antigens that are normally expressed only in male germ cells, e.g. NY-ESO-1, and 5) viral antigens, i.e. antigens that are derived from viral proteins expressed by tumor-inducing viruses, e.g. human papilloma virus protein E6.

**Immunotherapy Against Cancer** Several strategies to utilize the immune system for eradicating tumors have been tested. As the immune response is directed by various immune regulatory cytokines, investigators have used these in clinical trials. The “prototype” cytokine for these efforts has been IL-2, a cytokine that activates both T cells and NK cells \textit{in vivo}. In a series of clinical trials, pioneered by Steven Rosenberg and colleagues, showed that administration of IL-2 to cancer patients had beneficial effects in 16 \% of the patients\textsuperscript{57}. Although the clinical effect was relatively low, patients that responded to the IL-2 treatment showed signs of long-term protection from the disease compared to patients treated with conventional strategies, e.g. chemotherapy. However, high dose IL-2 treatment is associated with severe adverse effects, such as lung edema, which makes this treatment difficult to manage\textsuperscript{58}.

As described above, tumors display antigens that may be recognized by the immune system. After the development of successful immunotherapies based on
vaccination using mouse tumor models, the scientific community had great expectations on this approach to treat human cancers. However, after vaccination against various TAAs the overall conclusion is that only very low clinical response can be achieved in cancer patients. For example, a meta-analysis comprising thirty-two phase I/II vaccination trials reporting on 527 patients with advanced or metastatic colorectal cancer showed a very low frequency (<1 %) of clinical responses when immunotherapy was used as a treatment59.

Adoptive cell transfer therapy has recently been developed and clinical trials have demonstrated that it has great potential of causing tumor shrinkage. This treatment utilizes autologous tumor-infiltrating lymphocytes (TIL) that have been stimulated and expanded ex vivo and then transferred back to the patient60. In conjunction with non-myeloablative, lymphodepleting chemotherapy, this remedy has a ~50 % clinical response rate in patients with melanoma61. However, these clinical protocols are very cost- and labor-intensive and advanced techniques and skills are needed for this therapeutic approach, making it unfeasible for treating large numbers of patients.

In conclusion, there is a great need to improve active immunotherapy approaches and to boost the immune response in cancer patients at the same time, in order to overcome the tumor-induced immune dysfunction.

2.1. Cancer as a Chronic Inflammation

Cancer development is a multistep process in which normal cells acquire a series of mutations in tumor suppressor genes and proto-oncogenes, i.e. genes that control cell proliferation62. As first reported by Rous & Kidd, it is thought that viral or chemical carcinogens induce somatic changes that initiate a pre-neoplastic state before the transformation into a cancer cell63. This initiation comprises deoxyribonucleic acid (DNA)-damages that are irreversible and induce no phenotypic change of the pre-cancer cell15. In order for the pre-cancer cell into transform to a cancer cell, further insult is needed. These insults, including exposure to chemical irritants, factors secreted upon wound healing, hormones, chronic irritation and inflammation that induces cell proliferation, facilitate recruitment of inflammatory cells, which leads to additional DNA-damage15. These events induce additional mutations in tumor suppressor genes and proto-oncogenes, which ultimately lead to an immortal cancer cell.
Under physiological conditions inflammation is self-limiting and controlled tightly by the induction-versus-resolution balance (described in section 1.1 and 1.2). However, chronic inflammation at the tumor site is either due to a persistence of the initiating factors or a failure in the resolution of the inflammation\textsuperscript{15}. In a tumor two compartments can be identified: cancer cells and tumor stroma (surrounding tissue)\textsuperscript{64}. The tumor stroma provides vasculature to ensure proper gas exchange, supply of nutrients, and waste disposal, thus facilitating growth and sustenance of the tumor. The tumor stroma has traditionally been divided into three categories: blood vessels, inflammatory cells, and connective tissue. The inflammatory cells that are present in the tumor stroma mainly consist of macrophages and lymphocytes and these components of the stroma are seen in all cancers, but their extent varies between cancer types\textsuperscript{15}. For example, in carcinomas such as breast, stomach, and pancreas cancer, the tumor stroma accounts for over 90 % of the tumor mass. On the other hand, malignant melanoma and medullary carcinomas have minimal tumor stroma. Several similarities have been observed between tumor stroma generation and chronic inflammation: 1) initiation by leakage of plasma proteins or injured blood vessels\textsuperscript{64}, 2) extra-vascular blood clotting\textsuperscript{64}, 3) recruitment of inflammatory cells\textsuperscript{64}, 4) presence of pro-inflammatory molecules, such as TNF-α and histamine, and pro-resolution cytokines, such as TGF-β, IL-10 and VEGF\textsuperscript{15}, and 5) oxidative stress at the tumor site\textsuperscript{65}.

**The Link Between Inflammation and Cancer** Several lines of evidence indicate that infection and inflammation may induce cancer progression\textsuperscript{15,66-69} (Table II). Infection can induce chronic inflammation, thus facilitating oncogenesis through mechanisms described above. For example, a strong positive correlation between colon cancer development and inflammatory bowel disease, such as chronic ulcerative colitis and Crohn’s disease, has been observed\textsuperscript{15}. Hepatitis C virus infection is known to be associated with liver carcinoma\textsuperscript{70}, schistosomiasis increases the risk of bladder carcinoma\textsuperscript{71}, and *Helicobacter pylori* infection is the leading cause of stomach cancer\textsuperscript{72}. These observations indicate that inflammation is a key factor in tumor development and one may argue that by interfering with tumor-induced inflammation/resolution it could be possible to induce a functional immune response and in turn facilitate tumor eradication.
### Table II – Inflammatory conditions associated with cancer

<table>
<thead>
<tr>
<th>Inflammatory conditions</th>
<th>Associated Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestosis, silicosis</td>
<td>Mesothelioma, lung carcinoma</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>Lung carcinoma</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>Colorectal carcinoma</td>
</tr>
<tr>
<td>Lichen sclerosus</td>
<td>Vulvar squamous cell carcinoma</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>Pancreatic carcinoma</td>
</tr>
<tr>
<td>Reflux oesophagitis</td>
<td>Oesophageal carcinoma</td>
</tr>
<tr>
<td>Skin inflammation</td>
<td>Melanoma</td>
</tr>
<tr>
<td>Gastritis/ulcers</td>
<td>Gastric adenocarcinoma</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Mononucleosis</td>
<td>B-cell non-Hodgkin’s lymphoma, Burkitts lymphoma</td>
</tr>
<tr>
<td>AIDS</td>
<td>Non-Hodgkin’s lymphoma, squamous cell carcinomas, Kaposi’s sarcoma</td>
</tr>
</tbody>
</table>

Modified from Coussens & Werb\(^{15}\)

### 2.2. Tumor Escape

The “six hallmarks of cancer” is a concept of cancer progression where the cancer cell gains six characteristics: the cancer cell is capable of providing autocrine growth signals, ignoring growth-inhibitory signals, avoiding cell death, replicating without limits, sustaining angiogenesis, and invading tissues through basement membranes and capillary walls\(^{73}\). It has now also been proposed that a seventh hallmark should be included: avoidance of immune surveillance\(^{74}\). Numerous cell types have been proposed to be involved in immune surveillance of cancer, i.e. CD8\(^{+}\) and CD4\(^{+}\) T cells, NK cells, NKT cells, and IFN-producing killer dendritic cells\(^{75}\). The eradication of cancer cells can be mediated by direct perforin dependent lysis of tumor cells (by triggering of specific T cell receptors (TCR):peptide/MHC class I recognition by T cells or NK cell triggering by “missing-self” signals and/or stress-induced NK cell receptor ligands), and/or by cytokines, e.g. IFN-\(\gamma\)\(^{75}\). All of these mechanisms can be counteracted by the tumor.

#### The Rise and Fall of the Immune Surveillance Concept

Paul Ehrlich was the first to formulate the idea that the immune system could repress the frequency of carcinomas\(^{76}\). In the 1960\(^{\circ}\) with the development of inbred strains of mice, immunization against tumor-transplants showed the existence of tumor-specific antigens\(^{51,52}\). Sir Frank Macfarlane Burnet and Lewis Thomas incorporated this discovery into the formal hypothesis of ‘cancer immune surveillance’. Burnet stated:
‘It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence’ (cited from 76).

In 1970, Burnet defined the immune surveillance concept, based on speculations made by Thomas:

‘In large, long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character’ (cited from 76).

After the introduction of the immune surveillance hypothesis many experiments were conducted to investigate how suppression of the immune system in mice affected the spontaneous or carcinogen-induced tumor incidence. However, these studies did not unequivocally prove or disprove the hypothesis. For example, Rygaard et al. showed no differences between spontaneous tumor formations in nude mice compared to normal mice indicating that immune surveillance did not exist77,78. However, these experiments had pitfalls, which were mainly caused by the limited understanding of the nude mouse. For example, it is now known that nude mice are not completely immunodeficient as these mice have functional NK cells79, thus, immune surveillance may occur in these animals.

The Return of the Concept From the mid 1970s the immune surveillance as a concept gradually lost credibility. However, three key findings in the mid 1990s sparked a renewal of the concept. Utilizing knock-out mice that lack the IFN-γ receptor 1 (IFN-γR1−/− mice), it was shown that wild-type mice had lower incidence of chemically induced and spontaneous tumors80-82. Secondly, mice lacking perforin (perforin−/− mice), a component of the cytolytic granules of cytotoxic T cells and NK cells that is important in mediating lymphocyte-dependent tumor cytotoxicity, were more sensitive to methylcholanthrene (MCA)-induced tumor formation compared to wild-type mice82-86. Finally, studies using mice that lack recombination activating gene 1 or 2 (RAG-1−/−, RAG-2−/−) and can therefore not undergo TCR rearrangement further supported the existence of the immune surveillance hypothesis. Experiments in this model showed that lymphocytes not
only protected the host against formation of chemically induced sarcomas, but also prevented the development of spontaneous epithelial tumors\textsuperscript{87,88}.

Furthermore, epidemiological studies have reported a higher probability of developing non-viral tumors in immune deficient humans\textsuperscript{89,90}. A positive correlation between lymphocyte infiltration and increased survival of cancer patients has also been shown, arguing for a role of the immune system in the eradication of human tumors\textsuperscript{91-93}.

\textit{Skepticism Still Remains} So far, the studies supporting the role of immune surveillance have not formally proven that it does exist. These studies, e.g. increased susceptibility to cancer in mice lacking functional T cells and B cells (e.g. RAG-1\textsuperscript{-/-} mice), show the final outcome as tumor/no tumor. However, the formal proof, i.e. the eradication of small, un-palpable tumors by the immune system has not been shown. Further, the possibility that the tumor is a consequence of a secondary event, e.g. inflammation caused by an infection, cannot be ignored, as these mice are very susceptible to bacterial or viral infection.

A recent study by Willimsky & Blankenstein argued against immune surveillance\textsuperscript{94}. In this study a mouse model of sporadic cancer based on rare spontaneous activation of a dormant oncogene was developed. However, the tumors grew in spite of expressing the rejection antigen and the outgrowth of the tumor was paralleled with an increasing T cell tolerance to the rejection antigen. Thus, tumor cells did not evade the immune system by altering recognition i.e. loss of antigen. The outgrowth of tumor cells was initially due to tolerization of tumor-specific T cells and, in later stages of tumor development, tumor-induced immune suppression was the underlying process for cancer in this model.

Although several lines of evidence point towards the existence of an immune surveillance system, it has not been formally proven. Presently, there is no consensus on what would constitute unequivocal proof. One may also argue that the murine model experiments should be conducted in germ-free mice, enabling the investigation of the role of immune surveillance in oncogenesis without infections as a confounding factor.

The fact that immunocompromised mice develop more tumors than normal mice can be regarded as an indication of immune surveillance, not a proof for continuous eradication of cancer cells. In order to obtain proof-of-concept, one
must develop a mouse model in which newly formed cancer cells can be visualized. For example, one may use a mouse model with a dormant oncogene that, upon activation, will co-express a reporter gene, such as the luciferase gene, enabling detection of small, unpalpable tumors in living animals. If a causal relationship between cancer formation, induction of an immune response, and eradication of the newly formed cancer cells is observed, the model could pose a valid argument for the existence of immune surveillance.

While immune surveillance against non-virally-induced tumors still remains to be proven, evidence for immune surveillance against virally-induced tumors exists. For example, it has been shown that young women vaccinated against human papillomavirus before first exposure to the virus have decreased incidence of cervical intraepithelial neoplasia\(^95\), arguing for immune surveillance against virally-induced tumors.

### 2.2.1. Immunoediting and Tumor Escape.

Cancers develop despite a functional immune system. It has been shown that tumors that develop in immune compromised hosts, e.g. RAG-1\(^/-\) mice, are rejected upon transplantation into an immune competent recipient, but tumors developed in wild-type mice with a functional immune system grow when transplanted into syngenic wild-type mice\(^87\). Thus, tumors may be “sculptured” by the immune system promoting the outgrowth of tumor cell variants that are poorly recognized by immune cells. It is thought that the evolution of tumor cells into less immunogenic clones is due to the inherent genetic instability of the tumor cell. To conceptualize these new findings with the inclusion of the immune surveillance theory, Dunn et al. proposed the term immunoediting\(^74\). In essence, the model proposes that the immune system facilitates tumor progression by exerting immunological pressure on the tumor as it develops (see Fig 3). Immunoediting comprises of three phases\(^74\): 1) elimination, 2) equilibrium, and 3) escape. In the elimination phase, tumor cells are continually eliminated by the immune system. However, if a tumor cell acquires traits that enable survival, the immune system and the tumor enter a state of equilibrium where the immune system suppresses tumor growth, but is unable to eradicate the tumor. This causes high immunological pressure on the tumor cells and favors survival of cells with mutations that makes the cells less immunogenic, less sensitive to cell death, and have traits that can induce immune suppression. This is called tumor escape and
several mechanisms have been shown to be involved. One may divide the mechanisms into two categories; 1) immune selection and 2) immune subversion.

2.2.2. Tumor Cell Immune Selection

Tumor cells that have been able to survive the initial elimination process by the immune system (immune surveillance) and entered the equilibrium phase are constantly targeted by effector cells of the immune system. In a Darwinian selection process, tumor cells that are not recognized by the immune system or tumor cells that gain traits that renders them resistant to cell death will have a growth advantage.

Lack of Tumor Recognition In order for a tumor cell to be recognized and killed by CD8+ T cells (cytotoxic T lymphocytes (CTL)), interactions between peptide: major histocompatibility complex class I (peptide:MHC I) on the tumor cell surface and TCR on T cells must take place. Through the oncogenic process, cancer cells can evade recognition by the T cell through mutations, gene deletions or gene regulation, leading to downregulation of proteins which are important for the antigen presentation machinery (APM). For example, it has been shown that the transporter associated with antigen processing (TAP), which is responsible for loading peptides on to the MHC I molecule, and essential components in the immunoproteosome, i.e. the low molecular mass polypeptide (LMP)-2 and LMP-7, are defective in patients with uveal melanomas96 and bladder cancer97 due to mutations in their genes. The consequence of this may be total or partial loss of
peptide:MHC I on the cell surface of the cancer cell. In addition, studies from our group have shown total loss of the human leukocyte antigen (HLA) class I locus in ovarian carcinoma\textsuperscript{98} which may contribute to disease progression resulting in failure of active immunotherapy\textsuperscript{99}. Antigenic loss, or “epitope” loss, is another way by which tumor cells avoid recognition by the immune system\textsuperscript{100}. The rapid generation of new, genetically instable tumor cells creates an array of tumor cell variants that may lack a tumor antigen. Thus, the antigen loss variants may be able to proliferate unnoticed by the immune system.

**Lack of Susceptibility to Cell Death** Upon recognition, immune cells kill tumor cells by inducing apoptosis. There are two main ways of inducing apoptosis; 1) the death receptor signaling pathway and 2) the perforin/granzyme B pathway\textsuperscript{101}. Both pathways result in intracellular cysteine-dependent aspartate-directed proteases (caspase) activation, which leads to apoptosis. Tumor cells may prevent apoptosis by modulating these pathways. For example, it has been shown that the death receptor signaling pathway can be avoided by cells that release factors, such as soluble Fas\textsuperscript{102}, or autocrine secretion of Fas ligand (FasL)\textsuperscript{103}. TRAIL-mediated apoptosis may be evaded through expression of the decoy receptors TRAIL-R3 and -R4, which lack the intracellular domain for correct apoptosis signaling\textsuperscript{104}. In the perforin/granzyme B pathway, T cells or NK cells release granules containing the proteolytic enzyme granzyme B, which together with perforin induces apoptosis. It has been demonstrated that cancer cells can escape this type of killing by overexpressing an inhibitor of granzyme B\textsuperscript{105}.

In addition, apoptosis is regulated by pro-apoptotic and anti-apoptotic molecules. Bcl-2, a proto-oncogene encoding a mitochondrial protein, is a key element in blocking programmed cell death. Dysregulation of Bcl-2 function leads to an increased ability to withstand apoptotic stimuli in cancer cells leading to cancer progression. Bcl-2 over-expression in acute myeloid leukemia cells is associated with an increased recurrence rate and a significantly lower survival after intensive chemotherapy\textsuperscript{106}.

**2.2.3. Tumor Cell Immune Subversion**

Upon further immune selection of tumor cells, the tumor can evolve to subvert the immune system by utilizing normal physiological functions. Several mechanisms have been proposed to explain this tumor-induced immune dysfunction.
Inhibition of T cells by Tolerogenic DCs}

Mature DCs prime T cell responses, thus allowing naïve T cells to be activated and become effector cells which in turn eradicate infected\textsuperscript{107} or transformed cells\textsuperscript{108}. Mature DCs also direct the immune response to polarize into a T helper 1 (TH1) or TH2 type\textsuperscript{109–111} and improve the generation of T cell memory\textsuperscript{112}. To perform these tasks, DCs have to undergo differentiation and maturation\textsuperscript{113}. Maturation of DCs is induced by both bacterial products\textsuperscript{114,115} and pro-inflammatory cytokines, especially TNF-α\textsuperscript{116}. If an antigen is captured and presented by a DC that is not fully activated, thus lacking the proper co-stimulatory molecules for interaction with T cells, this results in the induction of T cell tolerance\textsuperscript{113}. It has been shown that DCs at the tumor site have an immature phenotype\textsuperscript{113}. In addition, tumor cells can modify immature DCs to induce T\textsubscript{reg}\textsuperscript{117} and facilitate T cell unresponsiveness\textsuperscript{118}. It has been suggested that tumor-derived factors, such as VEGF, granulocyte macrophage-colony stimulating factor (GM-CFS), IL-10, and TGF-β, may induce the accumulation of immature DCs, thus contributing to T cell tolerance towards tumor cells\textsuperscript{119}.

Immune System-derived Soluble Factors Associated with Tumor-induced Immune Dysfunction

The induction and resolution of an immune response is mainly regulated through soluble factors, such as cytokines (see section 1 for details). Normally, factors mediate the end of an inflammatory episode, e.g. TGF-β production in late stage of an inflammatory process, transforms the response from anti-pathogenic and tissue-damaging to promoting angiogenesis and tissue repair. However, the chronic state of inflammation in cancer patients facilitates the generation of immune suppressive soluble factors when the immune system is triggered, thus suppressing a potentially tumoricidal immune response.

CD4\textsuperscript{+} T\textsubscript{reg} have the ability to produce high levels of the immunosuppressive molecules IL-10 and TGF-β and under normal circumstances are important to counteract autoimmunity\textsuperscript{120}. There is strong evidence that CD4\textsuperscript{+} T\textsubscript{reg} are involved in the tumor-induced suppression of the immune system\textsuperscript{121}. CD4\textsuperscript{+} T\textsubscript{reg} that express forkhead box P3 (FOXP3) and high levels of CD25, also known as naturally occurring T\textsubscript{reg}, can be found in the tumor or in the circulation of cancer patients, and have been shown to promote tumor-induced immune dysfunction in several cancers. For example, an inverse correlation between the presence of T\textsubscript{reg} and survival has been reported in ovarian carcinoma\textsuperscript{122}. In addition, depletion of T\textsubscript{reg} with anti-CD25 antibodies leads to increased tumor rejection by T cells in mice\textsuperscript{123}.
The secretion of TGF-β may be a major obstacle for effective immunotherapy as recent studies show that TGF-β acts on CTLs to repress the expression of perforin, granzymes, FasL, and IFN-γ, all important for CTL function. It has also been shown in mice that neutralization of TGF-β with specific antibodies restores the expression of IFN-γ, which leads to tumor clearance in vivo. In addition, it has been shown that TGF-β down-modulates the expression of the activating receptors NKp30 and NKG2D on T cells and NK cells and induces expression of the inhibitory receptors CD94/NKG2A. Moreover, CD1d-restricted NK cells may induce immune suppression by mechanisms involving IL-13 and TGF-β. In humans, there seems to be evidence for a comparable paradigm. Decreased frequency and function of CD8+ memory T cells and TILs was noted in TGF-β rich cultures from patients in a melanoma vaccine study, indicating an immunosuppressive role of TGF-β in cancer patients. Additionally, Treg were found in high frequencies in peripheral blood of cancer patients as compared to healthy donor blood and at the tumor site in patients with invasive breast or pancreas cancers, where they are likely to promote tumor growth by inhibiting anti-tumor immune responses.

IL-10 modulates the function of several cells in adaptive immunity and is considered to be one of the main immunosuppressive molecules. IL-10 can stimulate Tregs to secret TGF-β, adding to the immune suppressive environment. It has also been suggested that IL-10 plays a direct role in the differentiation of naïve CD4+ T cells into Tregs. Additionally, IL-10 has been shown to be secreted by melanoma cells leading to impaired function of DCs. IL-10 also favors a TH2 polarization by decreasing the production of IFN-γ and IL-12 by CD4+ T cells. It has been shown that high serum levels of IL-10 correlate with poor survival in renal cancer. Additionally, it has been suggested that PGE2 induces Treg, potentiating the secretion of IL-10 and TGF-β.

Induction of Lymphocyte Cell Death in Cancer Lymphocyte homeostasis is regulated by the thymic output of naïve T cells and death of peripheral T cells that have completed their functions. It has been shown that this homeostasis is disturbed in cancer patients leading to rapid lymphocyte turnover and loss of tumor-specific effector cells. It has been observed that TIL and peripheral blood lymphocytes (PBL) exhibit a high frequency of cell death compared to other inflammatory sites. Furthermore, studies have demonstrated signaling defects in the T cell receptor complex molecule CD3ζ in cancer patients and...
absence or decreased expression of CD3ζ has been correlated with poor survival of oral carcinoma patients\textsuperscript{138}. It has also been shown that signs of cell death are paralleled with a decrease in CD3ζ expression, suggesting a shared mechanism of the two events\textsuperscript{139}.

**Deprival of Essential Nutrients in Cancer** As described above, limitation of essential nutrients is a frequently used mechanism to limit biological activity, e.g. an immune response. However, due to the chronic inflammatory state in patients with cancer, the deprivation of these nutrients leads to inhibition of tumor-reactive immune responses. Two enzymes, IDO and ARG1, have been suggested to exert the immune inhibitory effect.

IDO, an enzyme involved in L-tryptophan metabolism, catalyzes the rate limiting step in the oxidative breakdown of L-tryptophan. IDO has been shown to be involved in immune tolerance in the placenta, which protects the fetus against attack by maternal T cells and has lately been shown to exhibit immunosuppressive functions in the tumor-micro environments when expressed in DCs\textsuperscript{140}. Studies investigating L-tryptophan and its metabolites indicate that IDO is chronically activated in cancer patients\textsuperscript{141} and that IDO activation correlates with progression of cancer\textsuperscript{142}. It has also been shown that low L-tryptophan concentration in the blood correlates with decreased survival in patients with T cell leukemia or colon carcinoma\textsuperscript{142,143} and that several tumor types express IDO\textsuperscript{144}. The mechanism of immune suppression is probably a combination of two factors; 1) overexpressed IDO in DC or tumor cells, depleting L-tryptophan in the tumor-micro environment, thereby inhibiting cell cycle progression in lymphocytes\textsuperscript{37} and 2) the toxic metabolite kynurenine produced in the reaction mediated by IDO, which is capable of inducing apoptosis\textsuperscript{145}. For example, Uyttenhove et al. showed that overexpression of IDO in tumor cells intensified the tumor cells’ ability to grow more aggressively and that this correlated with a decrease of functional T cells at the tumor site\textsuperscript{144}. The study also showed that administration of IDO inhibitors reduced tumor mass and stimulated anti-tumor immune responses.

ARG1 is also involved in inhibiting anti-tumor immune response by depleting L-arginine\textsuperscript{35,146}. It has been shown that depletion of L-arginine downmodulates CD3ζ expression\textsuperscript{146,147} and impairs the expression of cyclin D3 and cdk4 in T cells, inhibiting downstream signaling leading to G0-G1 cell cycle arrest\textsuperscript{36}. A study
analyzing the activity of ARG1 in peripheral blood mononuclear cells (PBMC) of renal cancer patients showed a significant increase in ARG1 activity compared to healthy controls. The study also revealed a correlation between ARG1 activity and a decrease in CD3ζ expression in T cells and NK cells, indicating ARG1-dependent immune suppression of these cells. Several cell populations have been shown to be implicated in ARG1 activity in tumor-bearing hosts; e.g. 1) increased ARG1 activity has been detected in cancer cell lines of colon, breast, or prostate cancer, 2) CD11b⁺, CD14⁺ myeloid-derived suppressor cell (MDSC) and granulocyte expression of ARG1 in patients with cancer mediate CD3ζ downregulation, thus inhibiting T cell proliferation and cytokine production.

**Tumor Cell-specific Immunosuppressive Mechanisms** There are several additional, tumor-specific mechanisms that are utilized to suppress the immune system in tumor-bearing individuals. In addition to the mechanisms mentioned above, these include expression of FasL, ligands for inhibitory receptors on T cells and NK cells, and galectins on the tumor cells.

The Fas-dependent cell death pathway is triggered in cells expressing Fas by the interaction with its ligand, FasL. FasL expression has been detected in several cancer types, including colon, lung, and esophageal cancer. Interaction of T cells and tumor cells induces Fas-dependent cell death in T cells, thereby facilitating a “Fas/FasL counter attack”. However, the presence of Fas/FasL counter attack is controversial, for example studies have shown pro-inflammatory functions of FasL expressed on tumor cells.

Tumor cells express ligands for inhibitory receptors on T cells and NK cells that block effector functions of these cells. For example, many tumors express major histocompatibility complex class I chain-related A (MICA), a stress-induced molecule with immune stimulatory function. However, it has been shown that tumor cells may shed MICA, which can bind to the NK cell or T cell activating receptor NKG2D, thus inhibiting the function of this receptor. Soluble MICA has been detected in plasma of cancer patients in whom a downregulation of NKG2D on T cells and NK cells was seen.

Galectin-1, a member of the lectin family, can hamper the T cell response. It is now evident that tumors, including breast, prostate, and colon cancer express galectin-1. Furthermore, a negative correlation between galactin-1 expression, tumor progression and numbers of T cells in the tumor has been shown.
Studies indicate that galectin-1 suppresses T cells through 1) sensitization of T cells to Fas-mediated cell death\textsuperscript{163}, 2) inhibition of TCR signaling by downmodulation of CD3\textgreek{z}\textsuperscript{164}, and 3) suppression of pro-inflammatory cytokines\textsuperscript{165}.

**Immune Suppressive Inflammatory Effector Molecules in Cancer** The above mentioned mechanisms of tumor-induced immune dysfunction originate from physiological processes that aim to resolve an inflammatory response. It has been shown that effector molecules in the inflammation phase mediate immune suppression. ROS and RNS produced by myelomonocytic cells are two of the key effectors in the anti-microbial machinery\textsuperscript{166,167}. However, these reactive molecules have been shown to have adverse effects on the tumor-specific response. It has been shown that ROS are produced by splenic macrophages from tumor-bearing mice\textsuperscript{168}, macrophages isolated from metastatic lesions of human melanomas\textsuperscript{169}, and activated granulocytes derived from peripheral blood of cancer patients\textsuperscript{170}. The secretion of ROS by these cells induces loss of T cell and NK cell functions, including defects in receptor associated signaling molecules, such as CD3\textgreek{z}, defects in nuclear factor-\textgreek{kB} (NF-\textgreek{kB}) activation in T cells\textsuperscript{171,172}, as well as a reduced cytokine production in response to T cell stimulation\textsuperscript{171,173-175}. These phenomena are also noted in T cells from cancer patients\textsuperscript{176}. ROS-producing cells may be monocytes, macrophages, granulocytes, or immature myeloid cells and can be detected in the tumor-micro environment\textsuperscript{168,177-179}, or in the peripheral circulation\textsuperscript{170}. In addition, it has been shown that ROS may be produced by the tumor cells themselves, adding to the oxidative stress in the tumor-micro environment\textsuperscript{180-182}.

NK cells are extremely sensitive to ROS-induced dysfunction\textsuperscript{183}. ROS produced by granulocytes and monocytes have been shown to hamper NK cell function and viability as well as the expression of NCR on NK cells\textsuperscript{169,184-187}. The down-modulation of NCRs and other NK cell receptors has been observed in NK cells from patients with acute myeloid leukemia\textsuperscript{188}, as well as other chronic inflammatory diseases such as hepatitis C\textsuperscript{189}, and active human immunodeficiency virus (HIV) infection\textsuperscript{190}.

In murine models, MDSC inhibited IFN-\gamma production by CD8\textsuperscript{+} T cells when peptide epitopes were presented in conjunction with MHC class I on MDSC surface\textsuperscript{191}. This was not true for CD4\textsuperscript{+} cells, indicating the specific impairment of cytotoxic cells. In some studies, this inhibition required direct cell-cell contact and appeared to be dependent on ROS, particularly hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})\textsuperscript{192}. In patients with
advanced tumors, immature myeloid cells inhibited antigen-specific T cell responses, indicating an explanation for reduced T cell response in patients with a high frequency of MDSCs in the tumor environment.\textsuperscript{177}

NO is a free radical that at low doses regulates several biological functions, such as blood flow and smooth muscle relaxation. This molecule is also produced by macrophages at high levels to function as an antimicrobial agent.\textsuperscript{193} Studies indicate that NO at higher doses is involved in chronic inflammation, such as in cancer.\textsuperscript{194,195} High concentrations of NO can generate RNS, such as nitrogen dioxide ($\text{NO}_2^*$)\textsuperscript{196}. These reactive molecules cause damage to DNA, proteins, and lipids, leading to hampered cell functions, increased inflammatory response, and cell death.\textsuperscript{197} The main NO-producing enzyme in macrophages is iNOS. Several lines of evidence indicate elevated levels of RNS at the tumor site. In breast carcinoma, tumor-infiltrating macrophages express iNOS and studies have found the expression of iNOS in colon adenomas,\textsuperscript{199} ovarian cancer,\textsuperscript{200} and melanomas.\textsuperscript{201} NO has been shown to severely inhibit antitumor responses by suppressing T cell function.\textsuperscript{202} Additionally, NO produced by macrophages inhibits activation of signal transduction, thereby suppressing T cell responses.\textsuperscript{204}

In parallel, studies utilizing probes for oxidative stress have shown that patients with cancer display increased levels of oxidative stress. For example, elevated tissue levels of malondialdehyde and 4-Hydroxynonenal (4-HNE), which are markers for the presence of lipid peroxidation by ROS, correlate with the clinical staging of colorectal cancer.\textsuperscript{65} Furthermore, increased oxidative stress-induced DNA damage, measured by increased levels of 8-oxo-2'-deoxyguanosine (8-OxoGua), has been detected in lymphocytes of colorectal cancer patients.\textsuperscript{205} It has also been suggested that oxidative stress is present in the blood, as studies show decreased levels of antioxidants in the plasma of colon cancer patients.\textsuperscript{65,205}

In conclusion, chronic inflammation associated with malignant tumors deregulates the induction versus resolution phases of the inflammatory process, which in turn facilitates disease progression. Several mechanisms are speculated to be responsible for the immune suppression and figure 4 shows a simplified model of cancer-associated chronic inflammation. In consequence, in order to improve the immunotherapy regimens available today, a combinatorial remedy aiming not only to eradicate the tumor, but to reverse tumor-induced immune dysfunction, is needed.
Fig 4 - Chronic inflammation caused by a tumor.
Upon insult, in this case triggered by tumor progression, tissue-resident mast cells and macrophages become activated by products produced after tissue damage or necrosis of tumor cells, e.g. neuro-peptides, HMGB1, and formyl-peptides derived from mitochondria of damaged cells. As seen in physiological response to an insult, the activation leads to neutrophil infiltration. Thereafter, monocytes enter the site and differentiate into macrophages where neutrophils and macrophages secrete ROS and RNS. Next to enter the tumor site are tumor-specific T cells that recognize cells that express tumor-associated antigens (TAA) and kill the tumor cells. However, the tumor is not totally eradicated as tumor-escape variants survive the initial immune response. This in turn leads to further tumor growth and increased activation of the innate immune system triggered by the continued necrosis of tumor cells and tumor stroma. In consequence, neutrophils and macrophages are chronically activated and continue to secrete ROS and RNS. In parallel, tumor-specific T cells continue to enter the tumor site, but lose effector functions and go into apoptosis due to the immune-suppressive tumor-microenvironment, including low levels of nutrients, accumulation of immune suppressive cytokines, tolerization by immature DCs, and oxidative stress. In conclusion, the process described above leads to chronic inflammation characterized by an environment that is toxic and suppressive for cells that are capable of tumor eradication, such as T cells and NK cells. In addition, tissue repair processes, such as angiogenesis, will also be induced, thus facilitating further tumor growth.
3. Lymphocyte Cell Death

Cell death is an essential physiological process in higher organisms as cell homeostasis is maintained by a balance between proliferation and cell death\textsuperscript{206}. For example, selective cell death is crucial for proper organ development\textsuperscript{206} and, as described above, cell death of lymphocytes and neutrophils is a necessity for the resolution of the inflammation phase. Defects in the process will lead to severe disease, such as autoimmunity, degenerative diseases and immunodeficiency\textsuperscript{206}. To ensure proper regulation of this vital process, hundreds of proteins are committed to govern the pro- and anti-cell death signaling within cells\textsuperscript{207}.

As a simplification, cell death can be divided into 1) active cell death, i.e. apoptosis and 2) passive cell death, i.e. necrosis\textsuperscript{208}. Apoptosis is mediated by a cascade of events, triggered by cellular stress, that leads to cell death and removal of the cell without release of toxic substances into the surrounding tissue, thereby preventing inflammation\textsuperscript{207}. Apoptosis is characterized by morphological changes, such as chromatin and cytoplasmic condensation, DNA fragmentation, phosphatidylserine externalization and formation of apoptotic bodies\textsuperscript{208}. On the other hand, necrosis is a passive, accidental cell death that manifests by rapid loss of plasma membrane integrity, which leads to an uncontrolled destruction of the cell and evokes the inflammatory process\textsuperscript{207}. Recently, it has become clear that cell death in higher organism is more complex and cannot be completely explained through the two processes mentioned above; however a detailed explanation of these processes is beyond the scope of this thesis.

The machinery mediating apoptosis involves caspases\textsuperscript{209}. Humans express at least seven caspases that govern this process\textsuperscript{210}. These molecules may be separated into two categories 1) the initiator caspases 2, 8, 9 and 10, and 2) the effector caspases 3, 6 and 7\textsuperscript{211}. Moreover, the apoptotic process is mainly mediated through two pathways, 1) the mitochondrial pathway, i.e. intrinsic pathway and 2) the death-receptor pathway, i.e. extrinsic pathway (see Fig 5).

**Intrinsic Pathway** The intrinsic pathway of apoptosis is regulated through proteins that interact in the vicinity of the mitochondria\textsuperscript{212}. This pathway is governed by the Bcl-2 family of proteins. This protein family is divided into groups as they have opposing roles in cell death due to differences in the number of Bcl-2 Homology (BH) domains that the protein contains\textsuperscript{213}. The anti-apoptotic group,
having three or four BH domains, includes Bcl-2, Bcl-xL, and Bcl-w. Upon activation
or overexpression, these proteins hinder the cell from implementing the cell death
machinery. In contrast, the pro-apoptotic Bax-like proteins (Bcl-2–associated X
protein (Bax), Bcl-2 Homologous Antagonist Killer (Bak), etc), which have two or
three BH domains, and the BH3-only group of proteins (Bcl2-antagonist of cell
death (Bad), Bcl-2-interacting killer (Bik), BH3 interacting domain death agonist
(Bid) etc), which has one short BH3 domain, induce cell death. When the cell is
exposed to stress, e.g. starvation or oxidative stress, the balance may be shifted
from the anti- to a pro-apoptotic state through activation of genes encoding the
pro-apoptotic proteins or activating post-translational modifications of pro-
apoptotic proteins. This will lead to formation of pores in the mitochondria
triggering mitochondria depolarization, which leads to permeabilization and
release of cytochrome c into the cytoplasm of the cell.

To counteract unwanted pore formation in the mitochondria, the anti-apoptotic
proteins stabilize the mitochondrial membrane by interacting with the pro-
apoptotic proteins, thus blocking their apoptotic capacity. Cytochrome c is
released into the cytosol and, together with apoptotic protease activating factor 1
(Apaf-1) and pro-caspase 9, forms the apoptosome that in turn activates the
executioner caspase 3. Upon mitochondrial disruption, other effector
molecules, such as Second mitochondria-derived activator of caspases
(Smac)/direct inhibitor of apoptosis-binding (DIABLO), are released, boost the cell
death machinery after cytochrome c release by hindering the function of several
inhibitors of apoptosis proteins (IAP).

**Extrinsic Pathway** Death receptors of the TNF receptor superfamily include Fas,
TNF receptor, and TRAIL receptor. Upon ligation, these receptors associate with
adaptor proteins containing death domains to form the death-inducing signaling
complex (DISC), resulting in caspase 8 activation. In turn, active caspase 8
cleaves and thereby activates caspase 3. Caspase 8 also cleaves Bid, which
induces Bak and/or Bax incorporation into the mitochondrial outer membrane,
leading to mitochondrial membrane depolarization and release of cytochrome c as
in the intrinsic pathway. The activation of caspase 3 leads to 1) cleavage of the
inhibitor of caspase-activated DNase (ICAD), inducing chromatin and DNA
degradation, 2) cleavage of lamins resulting in shrinkage of the nucleus, 3)
degradation of the cytoskeleton, and 4) membrane blebbing due to cleavage of
p21-activated kinases\textsuperscript{221}. The cumulative effect of all these processes is cell death by apoptosis.

### 3.1. Oxidant-induced Cell Death

ROS have a dual function in cellular systems, as it has been demonstrated that they can both stimulate cellular signaling pathways and trigger loss of cellular functions and cell death\textsuperscript{222,223}. It has been shown that redox signaling is important in T cell function, as activation of the TCR induces ROS generation\textsuperscript{224}, which is necessary for downstream-signaling pathways\textsuperscript{225}. On the other hand, ROS may also induce cell death in lymphocytes\textsuperscript{226}. Thus, the outcome of exposure to ROS seems
Upon activation, neutrophils and macrophages undergo respiratory burst. Respiratory burst is a rapid chemical reaction, facilitated by phagocyte oxidase (Phox), to produce superoxide ($O_{2}^{−}$) according to the reaction\textsuperscript{227}: $\text{NADPH} (M^{n-1+}) + O_{2} \rightarrow \text{NADP}^{+} (M^{n+}) + O_{2}^{−}$. $O_{2}^{−}$ may convert, via superoxide dismutase (SOD), into hydrogen peroxide ($H_2O_2$)\textsuperscript{228}. In turn, myeloperoxidase (MPO) may convert $H_2O_2$ into singlet oxygen ($^{1}O_{2}$), and further non-enzymatically into ozone ($O_3$). MPO may also assist in the conversion of $H_2O_2$ into hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypoiodous acid (HOI)\textsuperscript{229,230}. In parallel, $O_{2}^{−}$ and $H_2O_2$ may non-enzymatically produce hydroxyl radical ($OH^{•}$). The above mentioned molecules are examples of reactive oxygen species (ROS). On the other hand catalase (CAT) may decompose $H_2O_2$ into $H_2O$ and $O_2$.

Furthermore, the primary source of reactive nitrogen species (RNS) is NO produced by macrophages\textsuperscript{231}. NO is generated by inducible nitric oxide synthase (iNOS) in the reaction\textsuperscript{231}: $L$-arginine $\rightarrow L$-citrulline + NO$^{•}$. Together with $O_{2}^{−}$, NO$^{•}$ may, non-enzymatically, produce peroxynitrite (ONOO$^{−}$). Chemical reactions involving NO$^{•}$ lead to oxidation, nitration (addition of NO$2$), nitrosation (addition of NO), and nitrosylation (addition of NO on cysteine residues) of cellular components\textsuperscript{231}.

Thus, activation of neutrophils and macrophages leads to the generation of ROS and RNS. These molecules are chemically unstable and may lead to damage of cellular structures, such as proteins, lipids, or DNA. Furthermore, these molecules may also act as second messengers in signal transduction, thereby affecting the cellular response to oxidative stress.

Adapted from Ohshima et al.\textsuperscript{228}
unpredictable and it may be argued that it leads to activation of different processes, such as cell death or cell proliferation, depending on the pro- and anti-apoptotic balance within the cell and/or the antioxidant capacity of the cell.

When studying T cell lymphomas as a model for normal T cells, it was demonstrated that T cell death involves caspase 3 activity as caspase inhibitors prevented cell death. These studies also showed loss of the mitochondrial membrane potential and release of cytochrome c upon H$_2$O$_2$ exposure and demonstrated that the intrinsic cell death pathway was predominant upon exposure to H$_2$O$_2$. Furthermore, T cells that were activated in vivo induced apoptosis via a Fas- and TNF-α-independent pathway and AICD of T cells was characterized by caspase-independent loss of mitochondrial membrane potential. In parallel, T cells that were activated in vivo induced apoptosis via a Fas- and TNF-α-independent pathway and AICD of T cells was characterized by caspase-independent loss of mitochondrial membrane potential. In parallel, T cells that were activated in vivo induced apoptosis via a Fas- and TNF-α-independent pathway and AICD of T cells was characterized by caspase-independent loss of mitochondrial membrane potential. In parallel, T cells that were activated in vivo induced apoptosis via a Fas- and TNF-α-independent pathway and AICD of T cells was characterized by caspase-independent loss of mitochondrial membrane potential.

**Counteracting Oxidative Stress** Under normal physiological conditions, damage caused by ROS is counteracted by 1) several enzyme systems, such as superoxide dismutase (SOD) and catalase (CAT), and 2) scavenger systems, including the glutathione system, the antioxidants vitamin C, carotenoids, and vitamin E. SOD catalyzes the dismutation of O$_2^-$ into O$_2$ and H$_2$O$_2$, while CAT decomposes 2 H$_2$O$_2$ into 2 H$_2$O and O$_2$. Taken together these enzyme systems act synergistically by efficiently clearing ROS through the overall reaction:

$$4 \text{O}_2^- + 4 \text{H}^+ \xrightarrow{\text{SOD}} 2 \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} \text{O}_2 + 2 \text{H}_2\text{O}$$

The glutathione system is a complex antioxidant system that consists of monomeric glutathione (GSH), disulfide glutathione (GSSG), and glutathione
peroxidase, which catalyzes the reaction $2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$, and glutathione reductase, which converts GSSG to GSH. The glutathione system is a key defense against $\text{H}_2\text{O}_2$ and other peroxides.  

In conclusion, oxidative stress may either 1) induce a positive signal leading to lymphocyte activation, 2) have no significant effect on signaling pathways, or 3) induce a negative signal, leading to loss-of-function or cell death of the lymphocyte. The outcome could be dependent on several factors, including the level of oxidative stress and the lymphocyte’s susceptibility to cell death. On the other hand, the outcome may also be dependent on the antioxidant capacity of the lymphocyte, protecting cells with higher levels of antioxidants from oxidative stress-induced loss-of-function or cell death.
Aims of the Thesis

To improve the cancer immunotherapy treatments of today, a deeper understanding of tumor-induced immune dysfunction is needed. This knowledge may lead to the development of new therapeutic strategies to reverse the immune suppression seen in cancer patients.

 Accordingly, the aims of this thesis are:

► To improve the understanding of mechanisms leading to tumor-induced immune dysfunction and its effects on the immune system; focusing on oxidative stress as a source of immune suppression.

► To develop new treatment modalities to minimize tumor-induced immune dysfunction; focusing on prevention of oxidative stress.
Results and Discussion

4. Part 1 - Differences in Susceptibility to Cell Death of Lymphocyte Subsets

ROS are known to be involved in phorbol myristate acetate (PMA)-induced death of neutrophils, HIV-induced death of T cells, death of pancreatic β cells, and neural cell death. However, the molecular events leading to oxidant-induced cell death of lymphocytes is not fully understood. ROS-induced lymphocyte cell death has primarily been studied using T cell lymphomas, but the effect of oxidative stress on primary lymphocytes has not been extensively investigated. By examining ROS-induced cellular events within the various primary lymphocyte subsets, one can distinguish differences in the responses of the lymphocyte cell types to oxidative stress. The results are critical to a more detailed understanding of the influence of oxidative stress on the individual components that constitute the cell mediated immunity, particularly against tumors.

4.1. Implications of Preferential Cell Death of CD8+ TEM (Paper I)

T cells are one of the main effector cell types used in immunotherapy against cancer. As described above, oxidative stress, caused by ROS that are produced by granulocytes and macrophages systemically or at the tumor site, has detrimental effects on T cell function and viability. It has been shown that ROS-exposure of T cells at physiological levels leads to impaired TCR signaling, inhibition of NF-κB function, and decreased cytokine production. H2O2-secretion has been proposed as one of the sources of oxidative stress and may be one mechanism leading to tumor-induced immune dysfunction. We have previously demonstrated that H2O2 suppresses TH1 cytokine production and that this suppression correlates with inhibition of NF-κB activity. Of interest, the suppression of cytokines was primarily seen in memory T cells.

T Cell Subsets T cells can be categorized into subsets according to their effector functions and the expression of cell surface receptors. In essence, T cells can be sorted into two main compartments, CD4+ and CD8+ T cells. CD4+ T cells (T helper cells) support and coordinate the adaptive immune response, thus facilitating the effector functions, i.e. antibody- or cell-
Box 2 – Basics of T Cell Subsets

T cell memory is a requirement for a functional adaptive immune system. Through clonal expansion and differentiation of T cells specific for an antigen, the individual can acquire a life-long protection against a pathogen. Naïve T cells that have undergone the positive and negative selection in the thymus enter the blood circulation, home to the secondary lymphoid organs, e.g. lymph nodes, and await activation by DCs carrying processed antigens from an inflammatory site. If the T cell receptor on a naïve T cell recognizes the MHC:peptide complex (signal 1) on the surface of DC, the DC expresses proper costimulatory receptors (signal 2), and the DC secretes stimulatory cytokines (signal 3), the T cell becomes activated and starts to proliferate. These T cells then enter the circulation and home to the inflamed tissue. When the infection is cleared the majority of T cells go into apoptosis through AICD or ACAD. However, some T cells survive and become memory T cells. If the pathogen infects the individual again these memory T cells swiftly become reactivated and facilitate protection.

T cells may be divided into subsets according to 1) the existence of effector function of the T cell and 2) phenotypic expression of cell surface receptors that allow cells to home to secondary lymphoid organs versus non-lymphoid tissues. Simplified, T cells (CD3+CD56-) can be divided into CD4+ and CD8+ T cells. CD4+ T cells mainly assist the adaptive immune response determining the appropriate effector functions to use: antibodies or CTLs. CD8+ T cells (CTL) can lyse target cells upon encountering a cell expressing the cognate antigen for its TCR. After clonal expansion of CD4+ or CD8+ T cells, some cells survive and become memory T cells. These memory T cells are divided into effector memory T cells (TEM) and central memory T cells (TCM). Upon re-activation TEM migrate to the inflammatory site and have the capacity for immediate effector function. On the other hand, TCM have modest capacity for effector function and preferably home to T cell areas of secondary lymphoid organs. Upon antigen stimulation TCM have a high capacity to proliferate and differentiate into effector cells in response to antigenic stimulation. Thus, T cells may be divided into different subsets using the phenotypic cell surface marker CD45RO, CD45RA and the functional cell surface marker chemokine receptor-7 (CCR7). The functional consequence of CD45RA or CD45RO expression on T cells is not fully understood. The expression of CCR7 on T cells facilitates homing to secondary lymphoid organs. By using these markers one may divide T cell subsets according to the following model:

<table>
<thead>
<tr>
<th>T cell compartment</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>CD4+</td>
<td>CD8-</td>
</tr>
<tr>
<td>Naïve</td>
<td>-</td>
</tr>
<tr>
<td>TEMRA</td>
<td>-</td>
</tr>
<tr>
<td>TCM</td>
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</tr>
<tr>
<td>TEM</td>
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</table>
mediated response. On the other hand, CD8+ T cells (CTL) are responsible for the direct lytic activity against target cells. Activated CD4+ or CD8+ T cells expand and subsequently the majority of T cells undergo apoptosis; however, some cells survive and differentiate into memory T cells. By using cell surface markers and correlating the expression of these markers to the function of T cells, Lanzavecchia and co-workers have functionally characterized the T cell subsets. T cells may be divided into naïve, early effector (expressing CD45RA) and memory subset (expressing CD45RO). Memory T cells can be further divided into two subsets, effector memory T cells (TEM) and central memory T cells (TCM), depending on their ability to 1) migrate to the inflammatory site or to secondary lymphoid organs and 2) perform immediate effector function or proliferate and differentiate into effector cells in response to antigenic stimulation. The TEM subset, which expresses CD45RO but lacks expression of chemokine receptor-7 (CCR7) and CD45RA, is characterized by the ability to home to an inflammatory site and perform effector function. In contrast, the TCM subset, which expresses CD45RO and CCR7 but lacks expression of CD45RA, is primarily located in secondary lymphoid organs and has a potent ability to proliferate upon re-activation.

In a previous study we observed that cytokine production in the memory T cell subset was decreased upon oxidative stress, but naïve cells remained functional. We proposed the hypothesis that the loss of function of memory T cells was due to a “pre-apoptotic” state caused by ROS-exposure. To this end, we developed an experimental setup resembling oxidative stress in vivo. In the previous study, H2O2 was added exogenously to T cells and after 10 minutes the cells were washed to stop the ROS-exposure. However, it may be speculated that ROS-exposure in vivo has a longer duration. Since we used a setup that had H2O2 present throughout the entire incubation period in this study, we believe that this system resembles the in vivo situation more accurately. Furthermore, we improved the model by including purification of T cells using negative sorting techniques, enabling a more detailed analysis of cellular events in the different T cell subsets.

**Differences in Sensitivity to ROS-induced Cell Death in T Cell Subsets**

In this study, we examined the disparities in viability and function following oxidative stress among the various T cell subsets. We conducted a detailed phenotypic analysis, using fluorescently labeled antibodies specific for surface markers together with viability staining using cell death markers. Large differences were noted between the T cell subsets when comparing sensitivity to cell death
induced by increasing levels of H$_2$O$_2$. We concluded that CD8$^+$ T cells are more sensitive to oxidative stress than CD4$^+$ T cells and that memory T cells, in each subset, are more sensitive than naïve T cells. Furthermore, when investigating the different memory T cell subsets, we found that the CD8$^+$ T$_{EM}$ subset is particularly sensitive to cell death caused by low levels of oxidative stress and that CD8$^+$ T$_{CM}$ were not as sensitive as CD8$^+$ T$_{EM}$, although substantial cell death in CD8$^+$ T$_{CM}$ was also seen.

By using a pan-caspase inhibitor we were able to characterize the mode of ROS-induced cell death in memory T cells. We found that by inhibiting caspases we were able to reduce H$_2$O$_2$-induced cell death, indicating an important role for caspases in the cell death process. After conducting detailed time-kinetic experiments analyzing the temporal relationship between depolarization of mitochondrial membrane potential, caspase 8, and caspase 3 activity, we concluded that the intrinsic cell death pathway is primarily responsible for cell death, as mitochondrial depolarization was observed prior to caspase 8 and caspase 3 activity. In summary, memory T cells, especially CD8$^+$ T$_{EM}$, are more sensitive to oxidative stress-induced cell death as compared to other T cell subsets and cell death is characterized by caspase activity induced by mitochondrial depolarization. These findings are schematically depicted in Figure 6.

**Fig 6 - Different susceptibility to H$_2$O$_2$-induced cell death of T cell subsets**

Upon exposure to ROS, CD8$^+$ T cells go into apoptosis at lower H$_2$O$_2$ concentrations as compared to CD4$^+$ T cells. In addition, in the CD8$^+$ T cell compartment memory T cells, in particular T$_{EM}$, are more sensitive to oxidative stress-induced cell death and more rapidly undergo apoptosis as compared to other T cell subsets.
Differences in Signal Transduction in the T Cell Subsets It is possible that ROS produced by the T cell itself may have an important role in signal transduction. The model of oxidative stress described in previous sections is an example of oxidative stress supplied by external sources, e.g. ROS produced in the oxidative burst of neutrophils. It is known that H₂O₂ can diffuse through the cell membrane and one may speculate that exogenously added H₂O₂ can exert its effects intracellularly by acting directly on intracellular components. It has been shown that ROS can act as an internal messenger affecting cellular events, such as cell death caused by T cell activation²²³. Although not investigated here, it is possible that H₂O₂ used in this study acts as a signal transduction molecule mimicking normal T cell activation signaling events, thus inducing AICD. In theory, memory T cells may be more susceptible to AICD following H₂O₂-exposure due to factors connected to differentiation of T cells into a memory phenotype.

Differences in Pro- and Anti-apoptotic Molecules Another possible explanation for the disparity of sensitivity between the T cell subsets may be related to differences in expression levels of anti-apoptotic (e.g., Bcl-2 and Bcl-xL) and pro-apoptotic (e.g., Bax, Bak, and Bim) proteins. Indeed, it has been shown that CD8⁺ T cells have elevated levels of Bcl-xL and Bax as compared to CD4⁺ T cells²⁵⁴, it is therefore possible to speculate that the differences in pro-apoptotic protein expression could explain the differences in susceptibility to cell death between CD4⁺ and CD8⁺ T cells. In addition, Akbar et al. have shown that memory T cells have decreased levels of the anti-apoptotic protein Bcl-2 as compared to naïve T cells²⁵⁵. Other reports have demonstrated that Bcl-2 and Bcl-xL levels in T cells decrease after activation³¹,²⁵⁶,²⁵⁷. It is possible that exogenously added H₂O₂ induces cellular stress or DNA-damage, which leads to activation and/or expression of pro-apoptotic molecules. The increase in pro-apoptotic components may then be sufficient to override anti-apoptotic components in memory T cells as these cells have lower levels of counteracting anti-apoptotic proteins compared to naïve T cells, resulting in preferential cell death of memory T cells.

Implication for Current Immunotherapy Preferential cell death of CD8⁺ T_{EM} by oxidative stress may be a contributing factor to tumor-induced immune dysfunction and may explain the difficulties in developing effective immunotherapy against cancer. For example, IL-2 stimulated, tumor-specific T cell lines from tumor-infiltrating lymphocytes used in an adoptive cell transfer settings are predominantly of the CD8⁺ T_{EM} subtype⁶¹,²⁵⁸ and it may be hypothesized that
these cells, upon homing to the tumor site following administration to the patient, may encounter an oxidative milieu resulting in their elimination by the mechanisms described in this study. These therapies might therefore benefit from concomitant administration of oxidative stress antagonists, thereby rescuing T cells from cell death and augmenting the anti-tumor response.

4.2. Antioxidants Rescue CD56 brightly NK Cells from Cell Death (Paper II)

Like T cells, human NK cells can be divided into subsets. For NK cells, this division is based on the levels of CD56 expression, which correlate with differences in their biological function. The two NK cell subsets, CD56dim and CD56 bright NK cells, have different patterns of responding to stimuli. CD56dim NK cells have the ability to exert immediate cytotoxic functions and CD56 brightly NK cells mainly influence the immune system by secreting cytokines upon activation. It has been shown that NK cells protect from infection and may be of importance for the eradication of tumor cells. However, as described above, several studies have concluded that NK cells are exceptionally sensitive to oxidative stress. Thus, the environment in cancer patients is likely to hamper NK cell-mediated anti-tumor immunity.

A number of studies have reported an altered ratio of CD56 dim to CD56 bright NK cells at the inflammatory site. Members of our laboratory have observed a decrease in CD56 dim NK cells in ascites of ovarian cancer patients (manuscript in preparation). It is, however, not known if this is due to aberrations in induction, proliferation, or recruitment of CD56 bright NK cells or due to a preferential death of CD56 dim NK cells at the tumor site.

Based on our findings of differential susceptibility to oxidative stress-induced cell death in T cell subsets, we hypothesized that the altered CD56 dim/CD56 bright ratio might be due to differences in sensitivity of NK cells to cell death upon exposure to oxidative stress. To address this question we developed an in vitro model comprising co-culture of purified NK cells with ROS-producing neutrophils. In parallel with the oxidative stress model that we developed in paper I, a detailed investigation of possible differences between the NK cell subsets was conducted.

Altered CD56 dim/CD56 bright NK Cell Ratio due to Differences in Antioxidant Capacity CD56 dim NK cells undergo cell death upon co-culture with activated neutrophils, as a consequence of H2O2 secreted by the latter. In contrast,
Box 3 – Basics of NK Cell Activation

NK cells are lymphocytes that are regarded as a part of the innate immune system. It has been shown that NK cells are important in the immune response against viruses and tumors. NK cells do not possess recognition receptors, such as TCR on T cells; on the contrary, NK cells are governed by a balance of stimulatory and inhibitory signals mediated by receptor:ligand interaction with the target cell. Thus, NK cell activation is regulated by which stimulatory and inhibitory ligands that are expressed by the target cell and what stimulatory and inhibitory receptors are expressed on the NK cell.

One important feature in the regulation of the NK cell is its ability to differentiate infected or tumor cells, that have low levels of MHC class I molecules, compared to normal cells. This is made possible by expression of several of MHC class I-binding receptors on NK cells. If the target cell lacks the ligand for these receptors the NK cell becomes active due to a reduced inhibitory signal in the interaction with the target cell, which leads to lysis of the target cell; i.e. missing-self recognition by NK cells. However, NK cells also express stimulatory and inhibitory receptors specific for ligands on the target cell that is expressed if the cell is subjected to stress, such as virus infection, DNA-damage- or carcinogenic transformation of a cell. If the target cell expresses high levels of these stress-induced ligands, this may override the inhibitory signal facilitated by MHC class I molecules leading to executing of cell death of the target cell by NK cells.
CD56^{bright} NK cells are almost resilient to cell death. The selective susceptibility of CD56^{dim} NK cells to cell death was verified in a series of experiments exposing NK cells to exogenously added H$_2$O$_2$. Of note, the shift in CD56^{dim}/CD56^{bright} NK cell ratio was only true for ROS as NO- or $\gamma$-radiation-induced cell death did not show the same pattern, indicating a unique role for ROS. A possible explanation for the differences in susceptibility to ROS-induced cell death may be a difference in antioxidant levels in the different NK cell subsets. Indeed, assessment of the ability of ROS to enter the cell, as well as the antioxidant capacity exhibited by lysate from the different NK cell subsets indicated lower levels of antioxidants in CD56^{dim} NK cells as compared to CD56^{bright} NK cells.

**Potential Explanation for Differences in Antioxidant Levels** An independent study in parallel also reported that CD56^{bright} NK cells are less sensitive to oxidative stress as compared to CD56^{dim} NK cells, substantiating the findings of our study. Thorén et al. demonstrated that apoptosis in both NK cell subsets is dependent on PARP activity and that the differences in antioxidant levels are due to increased levels of surface thiols in the CD56^{bright} NK cell subset. Furthermore, Hanna et al. have conducted a detailed mapping of gene expression in the different NK cell subsets and found increased levels of glutathione peroxidase 1 in CD56^{bright} NK cells, an enzyme that catalyzes the reduction of H$_2$O$_2$ and glutathione, thereby adding to the antioxidant capacity of this subset.

In conclusion, we suggest that the accumulation of CD56^{bright} NK cells at inflammatory sites, can be explained by a rapid loss of CD56^{dim} NK cells due to ROS-induced cell death. The increased cell death of CD56^{dim} NK cells as compared to CD56^{bright} NK cells results from lower antioxidant levels and leads to a preferential survival and accumulation of CD56^{bright} NK cells at the site of inflammation.

**4.3. Conclusion Part 1**

In this series of experiments we show a disparity between T cell and NK cell subsets in their sensitivity to oxidant-induced cell death. Strikingly, in both T cells and NK cells, the cells with the highest potential to mount an anti-tumor response are the most sensitive to oxidative stress, i.e. CD8$^+$ T$_{EM}$ and CD56$^{dim}$ NK cells. This skewed sensitivity to ROS-induced cell death may have a great impact on the
success of immunotherapy approaches utilizing these cells as primary effector cells.

The underlying mechanism for the differential sensitivity to ROS-induced cell death in subsets of T cells and NK cells is, however, not identical. Based on our finding that NK cell subsets display altered levels of antioxidant molecules, we conducted a similar analysis investigating the antioxidant capacity in T cell subsets (unpublished data). The experiments performed thus far show no, or marginal, differences in antioxidant levels between T cell subsets. The preferential cell death of CD8^+ T_{EM} and CD56^{dim} NK cells may have different mechanisms. Our data suggest that, in T cells, a difference in sensitivity to H_2O_2-induced cell death signaling is the main reason for the different outcome of ROS-induced cell death in the T cell subsets (Figure 7A). This might be due to excess activation signaling, i.e. H_2O_2 acting as a second messenger leading to AICD. Alternatively, H_2O_2 could induce cellular stress leading to cell death as memory T cells have lower levels of anti-apoptotic proteins. In contrast, the differences in sensitivity of NK cell subsets to ROS-induced cell death may be due to differences in antioxidant levels, leading to lower overall levels of oxidative stress in CD56^{bright} NK cells as compared to CD56^{dim} NK cells on exposure to identical levels of oxidizing substances (Figure 7B). Although not addressed in this study, there may nevertheless be differences in sensitivity to cell death signaling in NK cell subsets as NK cells can also undergo activation-induced cell death.

In conclusion, these studies propose the use of treatment modalities aimed at reducing oxidative stress in cancer patients in order to rescue CD8^+ T_{EM} and CD56^{dim} NK cells from ROS-induced cell death upon encounter of the oxidative milieu in the tumor-micro environment, thus enabling a more efficient anti-tumor response.
5. **Part 2 – Therapies to Reverse Oxidative Stress in Lymphocytes**

It is now clear that tumor-induced immune dysfunction substantially reduces the efficacy of cancer immunotherapy. In addition to immunotherapy, one may develop combinatorial approaches that target these immune suppressive mechanisms in order to counteract immune suppression. With regard to oxidative stress as a cause of lymphocyte cell death, there are two main approaches to introduce molecules that can decompose ROS and decrease oxidative stress: 1) administration of antioxidants or 2) utilizing antioxidant enzyme systems.

5.1. **Using Vitamin E to Reduce Oxidative Stress in Colon Cancer Patients** (Paper III)

**The Use of Oral Administered Antioxidants** Vitamin E is a lipid-soluble antioxidant that can be administered orally to cancer patients\textsuperscript{271}. Vitamin E protects cells from ROS and increases cell membrane stability when located in cellular membranes\textsuperscript{271}. We have previously conducted a small clinical trial...
comprising 13 colorectal cancer patients receiving a short-term (2 weeks) supplementation of 750 mg vitamin E with the aim to reduce oxidative stress and thereby improve the status of the immune system. Analysis of peripheral blood T cells from these patients showed an enhanced production of IL-2 and IFN-γ and an increase in the CD4/CD8 ratio. With regard to NK cells, studies have shown that vitamin E may enhance NK cell activity and can protect lymphocytes from ROS-induced DNA damage when added to co-cultures with PMA-stimulated monocytes. Furthermore, vitamin E together with trace elements, has been shown to enhance NK cell activity. Studies have also shown that vitamin E levels are decreased in colon cancer patients indicating a high turnover of antioxidants and a possibility to restore antioxidant levels by supplementation of vitamin E.

Supplementation of Vitamin E Enhances NK Cell Lytic Activity in Cancer Patients We conducted a detailed analysis of the function, phenotype and receptor expression of NK cells from seven patients with colorectal cancer (Dukes stage C and D) that had received vitamin E during a period of two weeks. Our data show a clear improvement in NK cell lytic activity after vitamin E treatment. The mechanism for the improved activity could not be established despite numerous experiments. We did however determine that the increased NK cell activity was not due to 1) increased numbers of NK cells or an increase in the proportion of the CD56dim NK cell subpopulation, 2) increase in perforin expression or an enhanced ability of NK cells to produce IFN-γ, 3) alteration in levels of CD4+ Treg, 4) alteration in viability of NK cells, or 5) alternations in the overall cytokine milieu in blood plasma. An increase in the expression of the activating NKG2D-receptor was noted in NK cells from all patients. In theory, upregulation of the NKG2D receptor may be a factor contributing to the increased cytolytic activity of the NK cells. However, one patient who did not demonstrate increased NK cell activity also had an increase in NKG2D expression reducing the possibility that upregulation of NKG2D is exclusively responsible for the increased NK cell activity observed in this study.

Potential Mechanism of Action of Vitamin E Supplementation Although we were unable to define the mechanism of its protective effect, vitamin E can reduce oxidative stress either through incorporation into the membranes of NK cells or by reducing the overall oxidative status in peripheral blood, thus indirectly
decreasing the oxidative stress on NK cells. Other antioxidant modalities have been shown to be effective in boosting the immune system. For example, administration of the antioxidant histamine together with IL-2 and IFN-α showed improvement in the anti-tumor response in melanoma patients\(^{277,278}\). In summary, we demonstrate that oral administration of an antioxidant can improve NK cell function and that cancer immunotherapy in conjunction with vitamin E may have a greater clinical efficacy.

5.2. **Arming T Cells with an Antioxidant Enzyme** (Paper IV)

**Using Gene Therapy to Introduce an Antioxidant Enzyme** Another way to increase the ability of lymphocytes to resist oxidative stress is to utilize antioxidant enzymes that normally protect cells from ROS-induced injury, such as SOD, CAT and enzymes of the glutathione system\(^{238}\). Gene therapy approaches have been used in other studies to improve lymphocyte function. For example, gene transfer of a chimeric GM-CSF–IL-2 receptor\(^{279}\) or the CD28 molecule\(^{280}\) into human CTLs have been shown to improve T cell function. Expression of telomerase gene in T cells has been demonstrated to prolong the lifespan of transduced cells\(^{281}\). Gene modification of lymphocytes is thus feasible and has even been successfully used to introduce tumor-specific TCR into primary T cells for adoptive therapy of cancer patients\(^{282}\).

Several studies have shown that the exogenously added antioxidant enzyme CAT can protect lymphocytes from oxidative stress\(^{169,170,192}\). We therefore developed a gene therapy approach utilizing a retroviral system to insert the CAT gene into primary T cells, based on the premise that CAT expression would rescue the T cells from ROS-induced suppression. This gene therapy approach has been successfully utilized in other disease models, such as hyperoxia-induced injury\(^{283}\), oxidative stress-induced pancreatic islet destruction\(^{284}\) and neural cell degeneration caused by ROS\(^{285}\).

**CAT Expression Rescues T Cells from Oxidative Stress** We were able to increase levels of functional CAT in CD4\(^+\) and CD8\(^+\) T cells by retroviral delivery of cDNA encoding for the human wild-type CAT gene. CAT transduced T cells were less sensitive to oxidative stress-induced loss of function and cell death as compared to control cells when exposed to ROS. These experiments proved that CAT expression in primary CD4\(^+\) or CD8\(^+\) T cells can rescue these cells from oxidative stress, giving “proof-of-principle” that a gene therapy approach using an
antioxidant enzyme can potentially reverse tumor-associated immune suppression resulting from ROS.

**Advantages of a Gene Therapy Approach** Gene transfer based immunotherapies in cancer patients is cost and labor intensive. However, this approach has several benefits, as there are established clinical protocols using retroviral systems to transduce tumor-specific T cell receptors and concurrent retroviral delivery of the CAT gene could be done without major modifications. Furthermore, oral administration of antioxidants has several limitations including inadequate permeation of the antioxidant into the tumor site, as well as adverse effects related to the high antioxidant levels needed to achieve appropriate *in situ* concentrations. The use of CAT transduced T cells ameliorates some of these issues since the cells can proliferate and actively home to the tumor site as well as persist in the patients, protracting the anti-tumor effect.

**5.3. Conclusion Part 2**

We have investigated two approaches for using antioxidants in an attempt to decrease oxidative stress. In Paper III, we demonstrate that oral administration of vitamin E induces an increase in NK cell lytic activity. In Paper IV we developed a gene therapy approach enabling expression of the antioxidant enzyme CAT in primary T cells. Both methods can be applied to reduce oxidative stress in cancer patients. Systemic administration of vitamin E increased the antioxidant levels in the patients, whereas CAT transduction of T cells specifically sustains T cells upon encounter of an oxidative milieu, such as the tumor-micro environment.
Concluding Remarks

The immune system needs to be in perfect synchrony in order to be able to perform its function. The immune system responds to infections with both innate and adaptive components in order to rapidly eliminate pathogens. The immune response is tightly regulated in order to avoid immunopathological consequences. This regulation is mediated through pro- and anti-inflammatory mechanisms that govern the outcome of the immune response.

The balance of the immune system in cancer patients is skewed towards chronic inflammation. This chronic inflammation is known to decrease the efficacy of immunotherapy approaches. Tumor-associated immune suppression results from several mechanisms, such as secretion of anti-inflammatory cytokines, increased cell death of lymphocytes, deprivation of essential nutrients, and oxidative stress. Studies have shown that oxidative radicals formed intracellularly may activate lymphocytes; however, at higher concentrations ROS may instead induce loss of function and cell death of T cells and NK cells. We have here shown that oxidative stress targets the most efficient tumor reactive cells, i.e. CD8+ TEM and CD56dim NK cells. Our findings might explain the limited clinical responses noted for anti-tumor therapy utilizing these cell types.

We have used two strategies in order to counteract ROS-induced immune suppression, 1) oral administration of antioxidants aimed at decreasing the systemic as well as the intratumoral oxidative stress or at increasing antioxidant levels in lymphocytes and 2) utilizing an enzyme, introduced by gene-transfer into lymphocytes, in order to increase their antioxidant capacity. Our results show that both approaches may be beneficial in the reversal of the oxidative stress-induced immune dysfunction in cancer patients.

Other modalities have been suggested to counteract tumor-mediated immune suppression caused by mechanisms other than oxidative stress. For example, Cheng et al. were able to improve the function of tolerized CD4+ T cells, whereas Wang et al. were able to activate, instead of tolerize, tumor-specific T cells by manipulating the signaling pathways in DCs using tyrosine kinase inhibitors. In
The combination of one or several of these approaches with immunotherapy treatments may lead to reversal of tumor-induced immune dysfunction and an improved clinical outcome.

Chronic inflammation in cancer patients creates a disadvantageous milieu for immunotherapy approaches. There are several modalities to reduce the immune suppression.

Antagonistic antibodies that block TGF-β can be utilized to reduce the suppressive effect of the suppressive cytokines. A decrease in the expression of cytokines in tumor-infiltrating lymphocytes can be achieved by either targeting the cytokine itself, e.g., by administration of anti-TGF-β mAb, or by targeting cell populations responsible for the secretion of the suppressive cytokines, e.g., depletion of Tregs by anti-CD25 mAb.

Inhibitory receptors on T cells, e.g., CTLA-4, can be targeted directly reducing the suppressive effect and improving the therapeutic efficacy of DC can be employed. Antioxidants can be utilized to reduce oxidative stress-induced immune suppression. The effect of suppressive cytokines can be reduced by utilizing antioxidants.

Removing immune-privileged areas of tumor can provide an improved environment for the immune system to function effectively.

Drugs can block NO production, thus reducing RNS levels, or inhibit enzymes responsible for de novo synthesis. Inhibitory receptors on T cells can be targeted directly reducing the suppressive effect and improving the therapeutic efficacy of DC can be employed.
addition, studies have shown that targeting inhibitory receptors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)\(^{288}\) and programmed death-1 (PD-1) receptor\(^{289}\), on T cells with antagonistic antibodies potentiates the anti-tumor immune response.

CD4\(^+\) T\(_{reg}\) are implicated in the tumor-induced immune dysfunction and one of the main immune suppressive mechanisms that CD4\(^+\) T\(_{reg}\) exert is secretion of TGF-\(\beta\). Several studies have shown that depletion of CD4\(^+\) T\(_{reg}\) with antibodies, such as anti-CD25, has beneficial effects on anti-tumor activity\(^{123}\). Furthermore, it has been suggested that neutralization of TGF-\(\beta\) by methods such as administration of an anti-TGF-\(\beta\) receptor antibody and drugs blocking the intracellular signaling of the TGF-\(\beta\) receptor\(^{290}\) may also lead to reversal of this suppression. Blocking enzymes responsible for deprival of essential nutrients at the tumor site, i.e. IDO and ARG1, may also improve T cell function. Indeed, Muller et al. have observed a synergistic effect combining small molecule that inhibits IDO with chemotherapy\(^{291}\). Studies have also shown that blockage of ARG1 inhibits tumor growth and that this effect is mediated by the immune system\(^{146}\). NO production by iNOS as a source of RNS has also been targeted in order to reduce tumor-induced immune dysfunction and it has been shown that administration of iNOS inhibitor reverses T cell suppression\(^{292}\). It has further been shown that co-administration of ARG1 and iNOS inhibitors has synergistic effects on anti-tumor therapy\(^{292}\).

In conclusion, it is evident that there are ways to reverse tumor-induced immune dysfunction. As depicted in figure 8, it is conceivable that by combining existing immunotherapy approaches with one or several methods of reversing tumor-induced immune dysfunction, an improved clinical response in cancer patients could be achieved.
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