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TUMOR-MEDIATED CHANGES IN THE IMMUNE SYSTEM OF CANCER PATIENTS – A BALANCING ACT BETWEEN SUPPRESSORS AND EFFECTORS

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“yes, but the problem is…”

ABSTRACT

Tumors and immune cells interact in many ways: immune cells can recognize and even kill tumor cells, while the tumor on the other hand can induce cells of the immune system to participate in tumor-mediated immune subversion.

We studied immunosuppressive effects that human tumors exert on immune effector cells, particularly T cells, by inducing suppressive myeloid cells and decreasing T cell functional capacity.

Increased numbers of myeloid-derived suppressor cells (MDSC) have been found in tumor-bearing individuals in response to cancer-derived factors. We characterized a $CD14^{+}HLA\text{-}DR^{-/low}$ MDSC population in patients with melanoma that could strongly suppress T cell function. Suppressive activity was dependent on cell-cell contact, arginase-1 expression, oxidative stress, and STAT3 signaling. Melanoma MDSC exhibited a mixed phenotype including markers of both mature and immature cells. Due to their monocyte-like characteristics, we wondered whether the presence of MDSC could interfere with the generation of monocyte-derived dendritic cells (DC) for vaccine use. We found that melanoma MDSC exerted a dose-dependent negative effect on DC quality. The removal of MDSC from monocytes prior to DC generation could therefore be advisable in order to improve vaccine efficacy in diseases where $CD14^{+}HLA\text{-}DR^{-/low}$ cells have been observed.

Tumor-mediated immunosuppression has mostly been studied in patients with advanced cancer, thereby under-representing the group of early-stage cancer patients that should have a better chance to mount anti-tumor immunity and benefit from tumor immunotherapy.
We found that even patients with early-stage breast cancer exhibit signs of tumor-induced immune modulation. Expression of the ζ-chain, an important transducer of activating signals in T and NK cells, was down-regulated in patients compared with controls, but normalized after surgical tumor removal. Loss of ζ-chain expression was detectable in the blood, but strongest in the tumor, suggesting it to be mediated by tumor-derived factors. Further, circulating T cells of breast cancer patients were more differentiated than those of controls and exhibited signs of altered homing capacity. Tumor-associated T cells were dominated by effector memory cells that showed signs of activation, but were accompanied by indicators of immunosuppression.

The findings presented here show that various mechanisms of tumor-mediated immunosuppression are active in patients with early- as well as late-stage cancers. Understanding such tumor-immune interactions is the first step towards the design and optimization of immunotherapeutic strategies for the treatment of cancer.

LIST OF PUBLICATIONS

I. **Poschke I***, Mougiakakos D*, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive $CD14^{+}HLA\text{-}DR^{-/low}$ cells in melanoma patients are $Stat3^{hi}$ and overexpress CD80, CD83 and DC-Sign. *Cancer Research 2010; 70(11):4335-45*

II. **Poschke I**, Mao Y, Adamson L, Salazar-Onfray F, Masucci GV, Kiessling R. Myeloid-derived suppressor cells impair the quality of dendritic cell vaccines. *Cancer Immunology, Immunotherapy. 2011, in press*

III. **Poschke I***, De Boniface J*, Mao Y, Kiessling R. Tumor-dependent down-regulation of the ζ-chain in T cells is detectable in early-stage breast cancer patients and correlates with immune cell function. *International Journal of Cancer. 2011, in press*

IV. **Poschke I**, De Boniface J, Mao Y, Kiessling R. Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early-stage breast cancer patients. *Manuscript*

**contributed equally*

RELATED PUBLICATIONS not included in this thesis

- **Poschke I**, Mougiakakos D, Kiessling R. Camouflage and sabotage: tumor escape from the immune system. *Cancer Immunology Immunotherapy 2011; 60(8):1161-71;* Review
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** contributed equally*

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LIST OF ABBREVIATIONS

APC	antigen-presenting cell(s)
ARG1	arginase 1
ASC	neutral amino acid transporter
ATP	adenosine triphosphate
ATRA	all-*trans* retinoic acid
CAT	cationic amino-acid transporter
C/EBPβ	CCAT-enhancer-binding protein β
CMV	Cytomegalovirus
COX-2	Cyclooxygenase 2
CTLA-4	cytotoxic T lymphocyte antigen 4
DAMPs	danger-associated molecular patterns
DC	dendritic cell(s)
DNA	desoxy-ribonucleic acid
EBV	Epstein-Barr Virus
Flt	fms-like tyrosine kinase
FoxP3	forkhead box P3
GCN	general control non-repressed
GITR	glucocorticoid-induced TNFR-related protein
GMP	good manufacturing practice
GM-SCF	granulocyte-macrophage colony-stimulating factor
G-SCF	granulocyte colony-stimulating factor
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
IDO	indolamine 2,3-dioxygenase
IL	interleukin
iNOS	inducible nitric oxide synthase
ITAM	immunoreceptor tyrosin-based activation motif
L-Arg	L-Arginine
Lin	Lineage
LMP	low-molecular mass polypeptide
L-NMMA	L-NG-monomethyl Arginine acetate
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MCP	monocyte chemotactic protein
MDSC	myeloid-derived suppressor cell(s)
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metallopeptidase
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κB
NK	natural killer
Nor-NOHA	Nω-hydroxy-nor-Arginine
NOX	NADPH oxidase
$ONOO^-$	peroxynitrate
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor

PG	prostaglandin
RANTES	regulated upon activation, normal T-cell expressed, and secreted
ROS	reactive oxygen species
SCF	stem cell factor
SLN	sentinel lymph node
SOP	standard operating procedure
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TAM	tumor-associated macrophage
TAP	transporter associated with antigen processing
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
T_{reg}	regulatory T cell
VEGF	vascular endothelial growth factor

1 INTRODUCTION

This thesis describes part of my research on interactions between tumors and the immune system in cancer patients.
The first section will introduce important aspects relevant to the presented articles and should help even those who do not work in the field of tumor immunology to understand the contents and implications of my work.
An introduction to basic immunology is beyond the scope of this thesis, but I will try to help everyone understand the main text by providing short and simple background information in the grey infoboxes. There will be many of these in the beginning, but they will get fewer, as the reader becomes an (almost) expert immunologist. For more information on basic immunology please consult a text book, for example 'Janeway's Immunobiology' [1].
Animal models, especially preclinical studies performed in mice, have been crucial for advancing our understanding of basic biology and immunology. However, distinct differences exist between the immune systems of humans and mice. Obviously, the long-term goal of cancer research should be to improve the health, survival and quality of life of humans and not to cure mouse cancer (which has already been achieved successfully by many, including several of my colleagues).
During the work on my thesis, I have put a strong focus on studying the immune system in cancer patients. I will therefore emphasize previously published human studies in the introduction and include the results of animal studies only where they are essential to our understanding of a concept.

1.1 A FEW WORDS ON CANCER

Cancer is characterized by uncontrolled cell growth. It is initiated by one aberrant cell in a specific organ, which with time grows into a tumor, usually developing the ability to infiltrate neighboring tissues and metastasize (=spread) to other organs.
The survival and proliferation of healthy cells is tightly regulated, but this control is lost in cancer cells due to the occurrence of at least one, but most often many, DNA mutations. Mutations occur randomly, for example due to exposure to carcinogenic chemicals, UV irradiation, or just mistakes in the cellular machinery. Each mammalian cell contains an elaborate system to detect and repair such mistakes in the DNA, and does not allow a cell to divide before the mistake has been fixed, even causing the cell to undergo

Apoptosis, or programmed cell death, is the organized death of a cell. It can be due to intrinsic reasons, such as irreparable problems during cell division, or induced from the outside, for example by cytotoxic cells that signal through death receptors or release caspase-activating molecules into the target cell. **Caspases,** so-called molecular scissors, are proteases that activate each other in cascades that depend on the apoptotic stimulus, and co-ordinate the apoptotic process. Apoptosis is characterized by chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies, meaning blebs of the cell enclosed by the cell membrane. Apoptosis is generally considered to be a non-immunogenic form of cell death, since little antigen is released from the cell during the process, however this notion is still the subject of ongoing discussions.

Angiogenesis is the formation of new blood vessels, usually by 'sprouting' from existing vessels. This is important during embryonic development, but rare in healthy adult tissues that are already equipped with functional vasculature. Once they reach a certain size, tumors need to induce angiogenesis in order to get a sufficient supply of nutrients. Tumor-induced angiogenesis is often disorganized, making tumor vessels leaky. Vascular endothelial growth factor (**VEGF)** is one of the important factors that promote angiogenesis and is targeted in cancer therapy by the anti-angiogenic drugs bevacizumab (Avastin®), sorafenib (Nexavar®), and sunitinib (Sutent®).

apoptosis if necessary. As a consequence, the most frequent mutations in cancer cells are within this very control system, or in genes encoding for molecules that drive survival or cell division and therefore can permit persistence of cells with defective genetic material. It is easy to imagine that once one such mutation has occurred, additional mutations can be introduced and transmitted to daughter cells with greater ease. If this process cannot be inhibited, the ultimate result is a cell exhibiting the traits described as the 'hallmarks of cancer': sustained proliferative signaling, resistance to cell death, induction of **angiogenesis**, replicative immortality, capacity to invade tissues and metastasize, and evasion of growth suppressive signals [2].

During my thesis, I have mostly worked with samples from melanoma and breast cancer patients. Breast cancer is rare in men, but the most common cancer in women, with a lifetime risk of developing breast cancer close to 8% for Swedish women. Melanoma is a cancer of pigmented cells, and skin melanoma is amongst the top 10 most common cancers in both men and women, affecting more than 2600 and killing nearly 500 individuals in Sweden in 2009 [NORDCAN Cancer Database].

Figure 1 shows that the incidence of both diseases has increased over the past decades, possibly due to population growth, improved detection methods, the increased age of the population, or lifestyle factors.

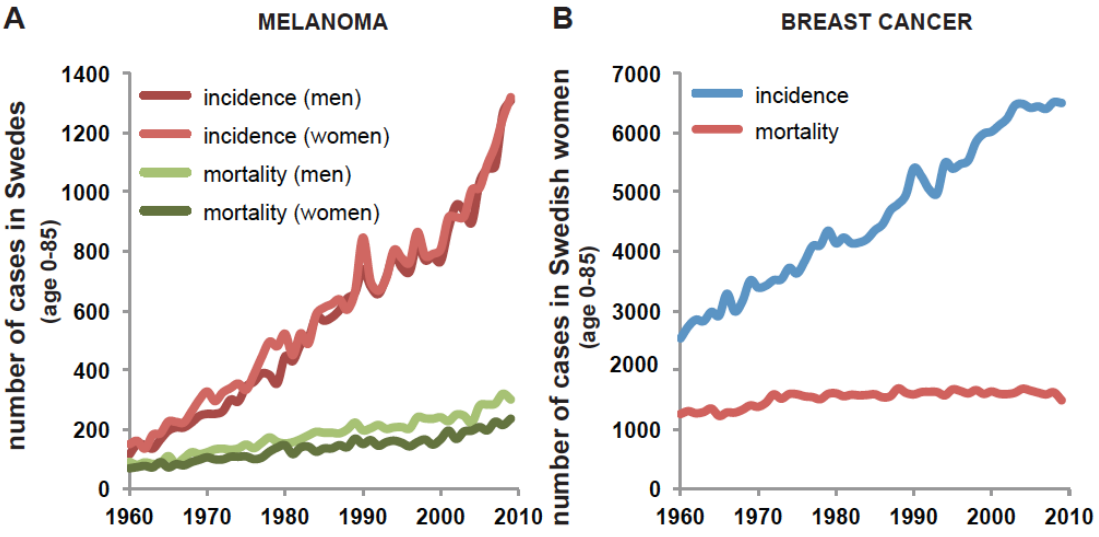


Figure 1: *Incidence (absolute number of newly diagnosed cases) and mortality of (A) melanoma of the skin and (B) female breast cancer in the Swedish population (age 0-85 years) between 1960 and 2009.*

Source: Socialstyrelsens statistikdatabas (http://www.socialstyrelsen.se/statistik/statistikdatabas)

Strikingly, though the incidence of both melanoma and breast cancer has more than doubled over the last 50 years, the mortality has remained nearly constant. This can be ascribed to greater prevention and screening efforts as well as to improved therapies. Unfortunately, a significant number of patients still die from their disease, stressing the need to develop additional curative treatments.
When thinking about cancer in the context of tumor-immune interactions, it is important to keep in mind that, despite of the presence of mutations, a cancer cell originates from within the body and will in many aspects resemble other cells from the same organ.

1.2 THE IMMUNE SYSTEM IN A NUTSHELL

The natural task of the immune system is to protect us from infections by bacteria, viruses, and parasites. This is quite a complicated affair, so I will give a brief, T cell-centric, and highly simplified overview, using the example of a viral infection.
The immune system consists of two arms: the innate arm, often termed the 'first line of defense', consists of cells equipped with pattern recognition receptors. These receptors recognize pathogen-associated molecular patterns, such as single-stranded viral DNA, and as a consequence induce activation and effector functions in innate immune cells. Usually this means that the intruding pathogen will be captured, internalized, and killed. At the same time, elements of the pathogen, so-called **antigens**, will be transported from the site of infection to secondary lymphoid organs, such as lymph nodes and the spleen, to be presented to the adaptive arm of the immune system. This is done by professional antigen-presenting cells (APC), of which dendritic cells (DC) are the most potent. The adaptive arm of the immune system consists of T and B lymphocytes. Each of these cells has a receptor specific for only one particular antigen. A T cell will therefore sample

An **antigen** is a molecule that can be recognized by the immune system. B cells recognize **epitopes** (parts) of proteins that are expressed on the cell surface using the B cell receptor. After activation and differentiation, plasma B cells will secrete soluble receptors (**antibodies**) specific for that same antigen. T cells mostly recognize **peptides** (small parts of proteins) between 8 and 24 amino acids in length. These peptides can be derived from any protein, but they have to undergo intracellular processing and loading onto MHC molecules (see p.5), which will then present the peptide on the surface of all cells (MHC I presentation to $CD8^+$ T cells) or of professional antigen-presenting cells (MHC II presentation to $CD4^+$ T cells). This means that the T cell receptor is actually not reactive to the antigen itself, but to the peptide epitope embedded into a specific MHC molecule. Some T cells also recognize lipid antigens on special antigen presentation molecules.

Cytokines are a family of soluble proteins that act as the 'messengers' of the immune system. When a cytokine binds its receptor, it usually induces an intracellular signaling cascade. Cytokines can act on the cell that has produced them (**autocrine**), on neighboring cells (**paracrine**), and on faraway cells (**endocrine**), as long as the target cell expresses the correct receptor. Cytokines usually have specific receptors, but many cytokine receptors share subunits, such as the common γ-chain that is part of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.

Chemokines, a subgroup of cytokines, are a family of small soluble proteins that bind to G protein-coupled transmembrane receptors. Many chemokines have more than one receptor. Chemokines are chemoattractants, meaning that cells expressing the correct receptor will move in response to a chemokine gradient. For example, chemokine receptors are important to help leukocytes that are circulating the blood at a quite high speed to stop and exit a blood vessel to exert their function, e.g., at a site of inflammation.

antigens presented by many different DC in the lymph node. If it encounters its cognate antigen, it will become activated, start dividing, and produce **cytokines**. Such activated T cells can then migrate to the site of infection in response to **chemokines** produced by the innate immune cells that first encountered the pathogen.

So called helper T (Th) cells, which are $CD4^+$, mainly produce cytokines, while $CD8^+$ 'killer T cells' can recognize and kill pathogen-infected cells that present e.g., viral antigens on their surface. Even natural killer (NK) cells can kill infected cells, though this is antigen-independent. NK cell activation is mediated through stress signals from the target cell and lack of inhibitory signals that prevent NK cells from attacking normal cells. Further, antibodies produced by activated B cells can bind and 'mark' the microorganism for destruction by innate immune cells, such as NK cells and macrophages.

Importantly, some of the activated, antigen-specific lymphocytes will persist as memory cells even after the infection is cleared and can be rapidly re-activated if we are infected by the same pathogen again.

2 CANCER AND THE IMMUNE SYSTEM

The primary task of the immune system is to protect us from infection. However, the receptors expressed by cells of the adaptive immune system are generated in a stochastic way and can therefore recognize antigens of any origin. It is therefore possible for lymphocytes to recognize antigens expressed by tumor cells, so-called tumor-associated antigens (TAA).

Here it is important to know that a number of tolerance mechanisms are in place to prevent autoimmunity. Newly generated T cells undergo 'education' in the thymus: T cells that have a T cell receptor (TCR) that reacts too weakly with **autologous** major histocompatibility complex **(MHC)** molecules 'die by neglect', while those that react too strongly with 'self' are killed due to negative selection. This mechanism, that prevents strongly self-reactive T cells from circulating through the body, is called 'central tolerance'.

Autologous means that something is from the same individual, while **allogeneic** means it is from another individual of the same species. For example, stem cell transplantation can be performed with autologous (the recipients own) or allogeneic (a donor's) bone marrow. **Xenogenic** describes the process of introducing something from another species, such as transplantation of a pig's heart valve into a human.

T cell activation requires at least two signals, i) the interaction of the TCR with an antigen presented in the context of an MHC molecule, and ii) co-stimulation by molecules that are only expressed on professional APC, such as DC, that are mature and activated. Co-stimulation will only be provided if an antigen has been taken up in a situation where '**danger signals**' were present. A T cell encountering antigen without co-stimulation will therefore not become activated, but instead tolerized or **anergic**. This is called 'peripheral tolerance' and prevents autoimmunity by T cells that have escaped negative selection.

Major histocompatibility complex (**MHC**) molecules are proteins that can present antigens to T cells. In humans, they are often referred to as human leukocyte antigen (**HLA**) molecules. There are nine genes encoding classical MHC molecules. HLA-A, -B, and -C are MHC class I molecules that present peptides derived from endogenous (the cell's own) proteins to $CD8^+$ T cells. This type of MHC molecule is expressed on every nucleated cell of the body. MHC I molecules are also important inhibitory receptors for NK cells. HLA-DP, -DQ and -DR are MHC class II molecules that present peptides derived from exogenous proteins, e.g., from bacteria that were captured outside the cell, to $CD4^+$ T cells. These molecules are only expressed on professional antigen-presenting cells such as DC, B cells, and macrophages. The non-classical MHC molecules include HLA-E, -F, and -G, as well as CD1 family members, and usually present defined epitopes, including peptides and lipids, and have mostly regulatory function.

Since tumor cells are mostly 'self', they should normally not be efficiently recognized by T cells unless their mutations result in i) new, non-self antigens, representing truly tumor-specific antigens or ii) changes in protein expression that make them different from normal cells or recognizable by T cells with low affinity TCRs. Furthermore, these tumor-derived antigens need to be accompanied by immune-activating danger signals.

In addition, some tumors, such as many cervical carcinomas, are associated with viral infections and therefore have 'tumor-specific' antigens that are actually derived from the virus and therefore unaffected by (central) tolerance.

With this in mind, the next chapter discusses what happens when the immune system does recognize a tumor and how some cells of the immune system are 'high-jacked' by the tumor to promote immunosuppression and cancer progression.

When T cells are stimulated in the absence of co-stimulation, they enter a state of **anergy**, where they are functionally unresponsive. This is part of a process called peripheral tolerance that prevents immunity to self antigens.

Danger signals are needed to alert the immune system when something is wrong, e.g., pathogens have entered the body. They activate the immune system and help to induce recruitment of additional immune cells. Danger signals can include i) danger-associated molecular patterns (**DAMPs**), i.e., molecules released from dying cells, such as free DNA and uric acid, ii) pathogen-associated molecule patterns (**PAMPs,**) as well as iii) cytokines released by activated immune cells.

2.1 THE CONCEPT OF TUMOR IMMUNE SURVEILLANCE

The concept of 'tumor immune surveillance' implies that the immune system can recognize and eliminate tumors. Sir F. McFarlane Burnet first postulated this theory in 1957 [3]. He believed that tumor immune surveillance was an "evolutionary necessity", because if it did not exist, everyone would die from cancer due to the large number of mutations occurring in the body on a daily basis [4]. This idea was further based on the observation that some cancer patients do not progress for prolonged periods of time, and some even exhibit spontaneous regression, sometimes in parallel to clearing an infection.

Early studies in mouse models showed that a vaccination with dead tumor cells could protect mice from a challenge with the same tumor cell line [5, 6]. This proved that there could be immune-mediated tumor rejection and that it was antigen specific, as vaccination did not work when a different cell line was used for the challenge. There is now ample evidence of tumor-specific T cells in the blood, bone marrow, and tumors of cancer patients [7-9].

It has also been shown that T cell infiltration into the tumor often correlates with a good prognosis [10-12], suggesting that the immune system can, to some extent, keep tumors in check.

It is of course difficult to ultimately prove the existence of tumor immune surveillance, crediting the immune system for a non-existing tumor. However, a large number of mouse models where one or several key immune mechanisms were deleted, has shown that such immunosuppressed animals often have a higher cancer incidence (reviewed in [13, 14]). Interestingly, loss of certain effector functions is often associated with susceptibility to particular types of cancer, suggesting that different malignancies are controlled by different immune effector mechanisms [15].

Epidemiological studies in humans under immunosuppression after organ transplantation show an increased cancer incidence in transplant recipients compared with the general population [16, 17]. It should be noted that many of the most commonly observed malignancies in immunosuppressed patients, such as lymphomas, have been associated with viral infections, however, even lung cancer and melanoma exhibited strongly increased hazard ratios [17].

Perforin and **granzyme B** are important effector molecules of cytotoxic T cells and NK cells. As the name suggests, perforin is a pore-forming protein that can make holes into the membrane of, or vesicles in, a target cell. These pores then allow the protease granzyme B to be released into the cell, where it cleaves caspases (see page 1) and thereby induces apoptosis.

A prospective study in Japan showed that individuals with high cytotoxic activity in their blood lymphocytes had a decreased risk of developing cancer later in life [22].

It is therefore possible that the ability of the immune system to recognize cancer cells can prevent tumor formation and influence tumor development. However, the fact that cancers commonly occur and frequently cause death, suggests that cancers often wins over the immune system.

Further, certain human immune deficiencies, such as **perforin** deficiency and **FasL** polymorphisms, are associated with an increased risk of developing lymphoma, lung and cervical cancer, among others [18-21].

FasL is an effector molecule of cytotoxic cells. Most cells in the body express its receptor, Fas, and receptor-ligand interactions induce apoptosis in the target cells via activation of the caspase cascade. After activation, both T and NK cells up-regulate Fas and therefore become susceptible to their own killing mechanisms. This is called '**fratricide**' and thought to prevent damage by over-activation. There are also some reports of tumors launching a '**counterattack**' on the immune system by expressing FasL and killing infiltrating T cells.

2.2 TUMOR IMMUNE ESCAPE

In 2011, an updated version of the 'hallmarks of cancer' was published, now including "avoiding immune destruction" as an 'emerging hallmark' [23].

It appears that the pressure exerted by tumor-specific immune cells ultimately shapes the tumor to become less immunogenic. The current model proposes three phases: the immune system initially controls tumor growth (elimination phase), then reaches a stage of equilibrium between immune-mediated tumor cell killing and novel mutations allowing tumor progression. Finally, the tumor will lose immunogenicity to an extent that it can escape from, and even suppress, the immune system [24]. Though this is proposed as a stepwise model, of course all mechanisms are at play simultaneously and the inherent genetic instability of the tumor will favor tumor progression by immune escape.

Figure 2 gives a schematic representation of the '3 E' model of immunoediting, showing the balance between tumor and immune cells during the elimination, equilibrium and escape phase.

Mechanisms of immune escape can roughly be divided into three categories:

i) lack of recognition, ii) lack of susceptibility, and iii) induction of immune suppression.

The proteasome is a multi-subunit complex containing several proteases. Its role in the cell is to degrade proteins. This is important for regulating protein levels, but also for destroying defective proteins. Proteasomal degradation is the first step of antigen processing and presentation and the resulting peptides can be presented to T cells on the cell surface after being loaded onto MHC molecules.

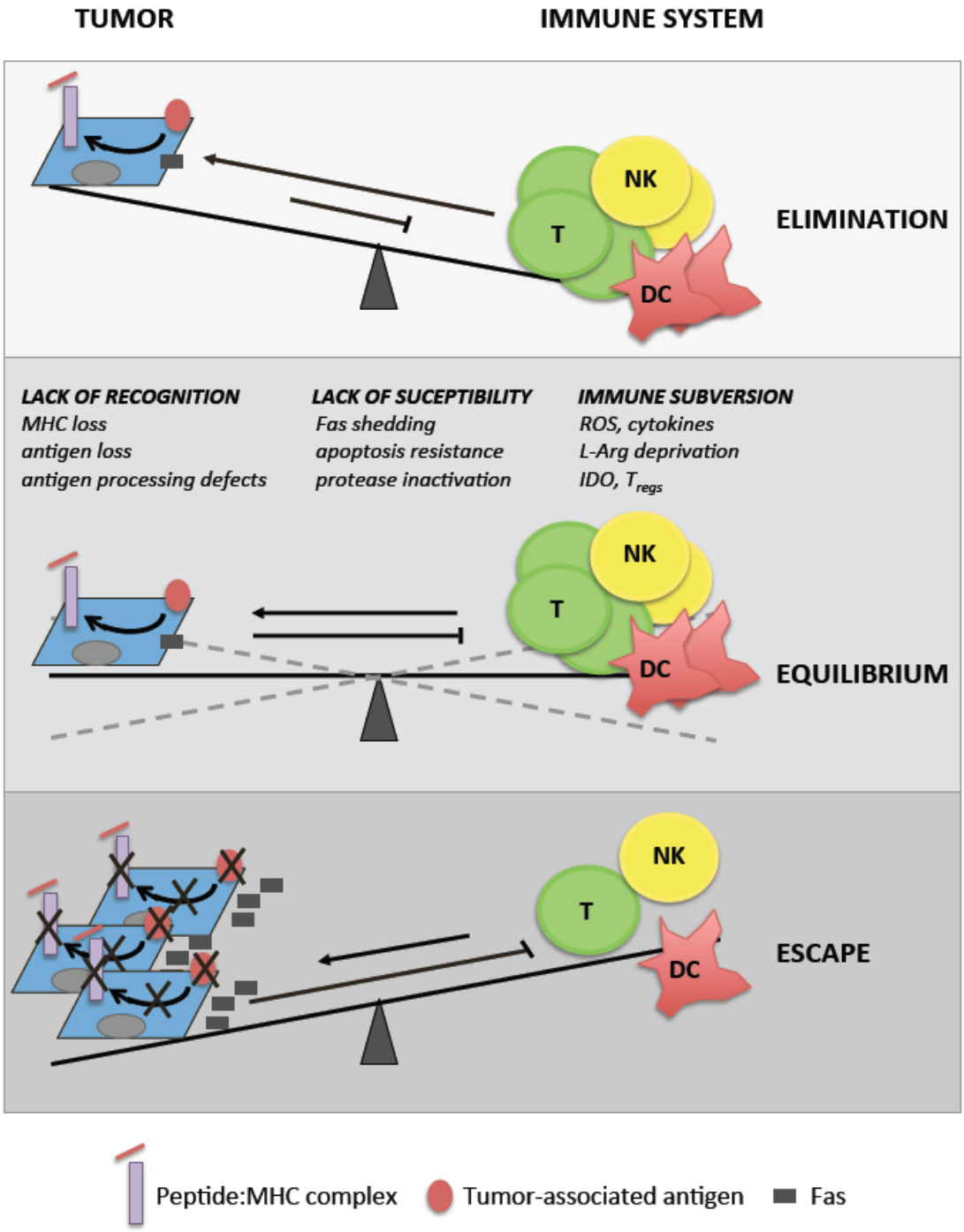


Figure 2: *The balance of tumor elimination, tumor immunoediting and tumor immune escape*

Lack of recognition can be due to the loss of antigens or to defects in antigen processing and presentation. Yee *et al.* reported a patient who initially responded well to immunotherapy, but eventually progressed [25]. Consecutive tumor biopsies revealed that at the start of therapy the patient's tumor expressed a range of tumor-associated antigens, but at progression, Mart-1, the antigen targeted by the therapy, had been lost, rendering the infused antigen-specific T cells useless. Similarly, there are many reports describing

defects in the antigen processing and presentation machinery [26-28]. Almost any of the molecules involved, including for example MHC, the transporter associated with antigen processing (TAP) and the **proteasome** subunits low molecular weight polypeptide (LMP)2 and LMP7, has been reported to be lost or down-regulated in cancer [29, 30]. This is particularly common in advanced disease and at metastatic sites, and has been shown to be a predictor of poor prognosis, e.g., in colorectal cancer, endometrial cancer, and melanoma [31-36].

Lack of susceptibility is often due to the tumor-intrinsic resistance to apoptosis, for example due to over-expression of anti-apoptotic molecules such as bcl-2 [2]. The cytotoxic mechanisms employed by T and NK cells ultimately result in the induction of apoptotic pathways, which are frequently turned off or counter-regulated in cancer cells [37]. Effector mechanisms that rely on receptor-ligand interaction, such as Fas-mediated killing or NK cell activation, can be rendered ineffective if the tumor cell down-modulates ligand expression or sheds key signaling molecules from its surface [38-40].

Induction of immune suppression by tumors can involve a myriad of molecules produced by the tumor itself or the tumor-mediated recruitment of immune cells with suppressive functions. These mechanisms are discussed in greater detail in the following paragraphs.

2.3 TUMOR IMMUNE SUBVERSION

Tumor-mediated immune subversion, or induction of immune suppression, can entail a large variety of mechanisms. Some of these, such as the secretion of suppressive cytokines, the generation of reactive oxygen species (ROS), and the presence of a nutrient-poor, hypoxic, and acidic environment, can be mediated by the tumor itself [23]. However, similar and additional functions can also be exerted by various cells of the immune system, which are found to accumulate locally and systemically in cancer patients.

2.3.1 Secreted factors

Cytokines. Tumors can secrete a variety of different cytokines, such as interleukin (IL)-10, transforming growth factor (TGF)-β, IL-6, and vascular endothelial growth factor (VEGF) (reviewed in [41]). Some of these cytokines act directly on surrounding tumor cells, but most of them target cells of the tumor stroma, including tumor-infiltrating immune cells.

Many of these secreted factors impair proinflammatory functions, for example by inhibiting DC differentiation and maturation [42], decreasing T cell cytotoxicity [43, 44], or up-regulating immunosuppressive molecules such as indolamine 2,3-dioxygenase (**IDO**) [45].

Indolamine 2,3-dioxygenase (**IDO**) is an enzyme catabolizing the reaction from L-tryptophan to N-Formylkynurenine. The depletion of tryptophan can induce a stress response in T cells, causing anergy and cell cycle arrest. Even tryptophan metabolites have been shown to exert immuno-suppression.

Surprisingly, even molecules with proinflammatory function, such as IL-6 and IFN-γ, are often pro-tumorigenic because they activate suppressive cell populations. This is described in greater detail in section 2.3.3.

Chemokines. Tumors can also secrete a number of chemokines. Some of them are directly associated with tumor progression and metastasis, such as CCL5, which has been shown to be expressed in breast cancer, but not normal breast tissue, and correlates with advanced disease stage and breast cancer progression [46, 47]. At the same time, chemokines can of course exert their normal chemoattractive function and promote tumor infiltration by myeloid and lymphocytic populations (see also 2.3.3).

Reactive oxygen species (ROS). The oxidative burst, i.e., release of high ROS concentrations, is a natural mechanism by which granulocytes and macrophages respond to pathogens. Reactive oxygen and nitrogen species also function as important messenger molecules that can act over short distances, e.g., within a cell. Due to their reactiveness they are usually tightly controlled and can be neutralized by cysteine-containing proteins that function as redox buffers.
The tumor microenvironment is known to be rich in ROS. There are only few reports about ROS production by tumor cells [48], possibly due to the difficulty of measuring ROS production by a specific cell type *in vivo* and the technical difficulty of determining ROS levels correctly in cultured cell lines. On the other hand, many tumor-recruited cells, such as myeloid-derived suppressor cells (MDSC), macrophages, and neutrophils, can release large amounts of ROS.
Unfortunately, many lymphocytes, especially the effector subsets of T and NK cells, are sensitive to ROS exposure, which can induce loss of function or apoptosis [49, 50]. Other T cell subsets, namely regulatory T cells (T_{regs}), exhibit a striking resistance to oxidative stress [51], possibly explaining how these cells can accumulate in cancer patients and the ROS-rich tumor microenvironment.

2.3.2 Regulatory T cells (T_{regs})

A great number of publications have shown that T_{regs} are increased in many different cancers and often correlate with poor prognosis (reviewed by [52]). There are several major T_{reg} subtypes: natural T_{regs} are $CD4^+CD25^+CD127^{low/neg}$ and express the transcription factor forkhead box P3 (FoxP3). Their suppression is mediated by contact-independent mechanisms such as the secretion of IL-10 and TGF-β, and cleavage of extracellular adenosine triphosphate (ATP), or contact-dependent mechanisms, mediated for example by membrane-bound TGF-β. FoxP3 expression is currently the most reliable marker for natural T_{regs}. However, like other proposed T_{reg} markers, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced TNFR-related protein (GITR), FoxP3 can be transiently expressed even by activated T cells. The lack of a truly unique marker makes the study, isolation, and therapeutic targeting of T_{regs} difficult.

Conventional non-suppressive T cells can be converted into T_{regs} when stimulated under tolerogenic conditions. Induced T_{regs} include Tr1 cells that produce IL-10, and Th3 cells that mostly suppress via production of TGF-β. There are even $CD8^+$ T_{reg} subsets, which are less well studied. T_{regs} can suppress proliferation and function of B and T cells, as well as DC differentiation [52]. In addition to the production of suppressive cytokines such as IL-10 and TGF-β, T_{regs} can exert suppression via consumption of IL-2 and ATP, and cysteine withdrawal, disturbing the redox balance of the target cell. APC that have encountered T_{regs} provide less co-stimulation and up-regulate IDO, inducing anergy in the antigen-specific T cells they interact with.
The main physiological role of T_{regs} is the prevention of autoimmunity, strikingly apparent in patients with FoxP3 mutations that develop severe autoimmune disease [53].
In cancer patients, an increased $T_{reg}/T_{effector}$ ratio is often observed in the blood [54-56], in tumor-draining lymph nodes [54], and in the tumor [54, 55, 57-59]. This increase in T_{reg} frequency and their infiltration into the tumor tissue, as opposed to peritumoral localization, has been shown to predict poor prognosis in a number of diseases [60, 61].
Interestingly, T_{regs} have also been shown to have a beneficial effect in some malignancies, probably due to their dampening effect on the proinflammatory mechanisms in some cancers. Recent mouse models demonstrated that the timing of T_{reg} activity might be essential, as T_{regs} favored tumor immune escape at an early stage, but did not affect outcome when depleted later during tumor progression [62]. In another tumor model, depletion inhibited or accelerated tumor growth when applied either early or late, respectively [63].
Therapeutic targeting of T_{regs} has achieved some clinical success. Certain types of chemotherapy have been shown to reduce the levels of circulating T_{regs} [64, 65], though the efficacy of the frequently used alkylating agent cyclophosphamide in this respect has been questioned [66]. A monoclonal antibody for the targeted delivery of diphtheria toxin to $CD25^+$ cells could deplete T_{regs} and improve the response to anti-cancer vaccines [67, 68]. In 2011, a monoclonal antibody targeting CLTA-4 was approved by the US food and drug administration for the use in melanoma patients. Though CTLA-4 is frequently expressed on T_{regs}, it is also up-regulated on activated T cells and its blockade by the antibody might act by preventing negative signals to conventional T cells rather than by acting on T_{regs} as originally intended [69] (see even chapter 2.3.4.2).
When studying T cell responses in cancer patients, the suppressive presence of T_{regs} has to be taken into consideration. Suppression exerted by T_{regs} might mask antigen-specific responses that become detectable only after T_{reg} depletion [70].

2.3.3 Myeloid-derived suppressor cells (MDSC)

2.3.3.1 Definition

MDSC are a heterogeneous population of myeloid cells with suppressive activity, containing precursors of granulocytes, macrophages, and DC.
The acknowledgement of these cells as important mediators of immune suppression has emerged relatively recently and the term 'myeloid-derived suppressor cells' was coined first in the year 2007 by a number of experts in the field [71].
MDSC have become a hot topic in recent years, with the number of publications yielded in a Pubmed search for the term 'myeloid-derived suppressor cell' shooting from 38 in 2007, to 60 in 2008, 82 in 2009, 136 in 2010, and over 130 already by the middle of 2011.
The role of MDSC in different diseases is now being thoroughly investigated and our understanding of their suppressive mechanisms has significantly increased. As a result, everybody likes to include MDSC in their immunomonitoring, though many probably fail to correctly identify their MDSC population of interest due to the phenotypic heterogeneity of human MDSC and the lack of functional studies.

2.3.3.2 MDSC phenotype

In the mouse, MDSC can be defined as $CD11b^{+}Gr1^{+}$ cells with suppressive function. Graded measurements of Gr1 expression or the use of two different Gr1 epitopes help to identify $CD11b^{+}Gr1^{high}$ ($CD11b^{+}$Ly-$6G^{+}Ly6C^{low}$) granulocytic MDSC, that are CD49d negative, and $CD11b^{+}Gr1^{low}$ ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) monocytic MDSC that express CD49d [72, 73]. Expression of the IL-4R α-chain (CD124) [74], the monocytic marker CD115 [75], low levels of the macrophage marker F4/80 [76, 77], and surprisingly also the stimulatory receptor CD40 [78] have also been suggested as markers for MDSC, though they are not unique and mostly lack relevance for identifying the suppressive population.
In humans, phenotypic characterization of MDSC is even more difficult. A great number of MDSC phenotypes has been described in many different human cancers. Some of these overlap at least partially, while others are mutually exclusive. Table 1 summarizes MDSC phenotypes described in human malignancies to date.
In 1995, Pak *et al.* were the first to describe human MDSC, which they found infiltrating tumors and metastatic lymph nodes of head and neck cancer patients. These cells were $CD34^{+}$ myeloid precursor cells and accumulated in response to GM-CSF secretion by the tumor [79].
Though this has not been tested in all studies, most human MDSC probably express both CD11b and CD33 and have low or absent expression of the MHC class II molecule HLA-DR.
Many reports describe MDSC that are $CD33^{+}$HLA-$DR^{-/low}$ and lineage (Lin) negative, meaning that they do not express CD3, CD19, CD56, or CD14, markers characteristic of T, B or NK cells and monocytes, respectively. This type of MDSC appears to be granulocyte-like and often suppresses via the

***Table 1**: MDSC phenotypes described in peripheral blood of cancer patients*

Phenotype	Suppressive Mechanism	Suspected/Excluded Mechanisms	Cancer	Ref
GRANULOCYTIC				
Lin^-DR^-	unknown	soluble factor, not NO	HNC, NSCLC, breast cancer	[80]
$Lin^-HLA\text{-}DR^-CD33^+$	**ROS, NO**		RCC*	[81]
	not tested	*in vitro* generated MDSC produce IL-10 + TGF-β	glioma	[82]
	not tested		RCC, ovarian cancer, bladder cancer, thymoma, leiomyosarcoma, biliary tree adenocarcinoma, rectal adenocarcinoma, small-cell lung cancer and NSCLC*, pancreatic cancer, thyroid cancer, melanoma, breast cancer, colon cancer, esophageal cancer, sarcoma, gastric cancer, HNC*, gall bladder cancer, prostate cancer, adrenocortical cancer, appendix cancer, HCC*, carcinoid, unknown primary	[83-87]
$Lin^-HLA\text{-}DR^-CD33^+CD11b^{-/low}$	not tested		colorectal cancer, breast cancer	[88]
$Lin^-HLA\text{-}DR^-CD33^+CD11b^+$	not tested	ARG	pancreatic cancer, esophageal cancer, gastric cancer	[89]
$CD11b^+CD33^+CD14^-$	not tested		RCC, soft tissue sarcoma, pancreatic cancer	[90,91]
$CD15^+$	**ROS**		pancreatic cancer, colon cancer, breast cancer	[92]
$CD15^+CD14^-$	**ARG**	trend for ROS	RCC	[93]
$CD15^+CD11b^+CD14^-$	not tested	Arg	RCC	[94]
$CD15^+CD11b^+CD33^+HLA\text{-}DR^-$	not tested		breast cancer	[95]
$CD11b^+CD33^+$	not tested		NSCLC	[96]
$CD11b^+CD33^+CD14^-$	not tested	ROS	HNC	[97, 98]
$CD11b^+CD33^+HLA\text{-}DR^-$	not tested		RCC	[99]
$CD11b^+CD33^+CD15^+CD14^-$	**ARG, iNOS**		NSCLC	[100]
$CD11b^+CD14^-$ $CD14^-CD66b^+$	**ARG**		RCC	[101]
$SSC^{high}CD66b^+$	not tested		HNC, bladder cancer, urothelial cancer	[102]
MONOCYTIC				
$CD14^+$	**ARG, iNOS**		HNC, multiple myeloma	[103]
$CD14^+HLA\text{-}DR^{-/low}$	**IL-10**		ovarian cancer (ascites)	[104]
	TGF-β	not ARG, not iNOS	melanoma	[105,106]
	ARG	ROS, STAT3	melanoma	[107]
	ARG	induction of T_{regs}	HCC	[108]
	unknown	NKp30 mediated NK cell suppression, not Arg/iNOS/IDO	HCC	[109]
	ARG		B cell non-Hodgkin lymphoma	[110]
	not tested	IL-10	prostate cancer	[111]
	not tested		colon cancer, mesothelioma, melanoma, fibrosarcoma, osteosarcoma, RCC, cervical cancer, pancreas cancer, NSCLC, glioblastoma, multiple myeloma, melanoma	[112-115]
UNKNOWN				
$CD3^-HLA\text{-}DR^-CD33^+$	not tested	STAT3 dependent	breast cancer, colon cancer, prostate cancer	[116]
$CD34^+$ (myeloid progenitor)	not tested		HNC	[79]
BOTH PRESENT				
$CD14^+$, PMN	not tested		colon cancer, melanoma	[117]
$CD11b^+CD15^{high}CD33^{low}$ $CD11b^+CD15^{low}CD33^{high}$	not tested	T_{reg} induction	bladder cancer	[118]
$CD33^+HLA\text{-}DR^-CD15^+$ $CD33^+HLA\text{-}DR^-CD14^-CD15^-$ $CD33^+HLA\text{-}DR^-CD14^+$	not tested	ARG	glioma	[119]
$CD11b^+CD15^+CD33^+HLA\text{-}DR^-$ $CD14^+HLA\text{-}DR^-CD33^+$	not tested		pancreatic, esophageal, colon, gastric, appendix, gall bladder cancer, cholangio-carcinoma, HCC	[120]
$CD14^+HLA\text{-}DR^+CD33^+$ $CD14^-HLA\text{-}DR^-CD33^+$	not tested		squamous cell carcinoma	[121]

**RCC – renal cell carcinoma, HCC – hepatocellular carcinoma, NSCLC – non-small cell lung cancer, HNC – head and neck cancer*

production of arginase I (ARG) or ROS, though this was not experimentally validated in all studies.
Some have observed neutrophilic MDSC with more distinctive granulocyte characteristics such as expression of CD15 and the characteristically lobulated nucleus. It is possible that this population has escaped the attention of many, since neutrophils are short-lived, highly sensitive to freezing, and mostly excluded when peripheral blood mononuclear cells (PBMC) are isolated by gradient centrifugation as is routinely performed in many laboratories. However, activated granulocytes (and MDSC) have been shown to co-purify with PBMC [92, 101, 102] and have increased resistance to apoptosis [101, 102], though it is still likely that frequencies of such neutrophilic MDSC are often strongly underestimated due to sample handling. Further, a number of studies has detected expression of the granulocyte markers CD15 or CD66b in $(Lin^-)HLA\text{-}DR^-CD33^+$ cells [93, 95, 96, 118-120], indicating that these populations overlap at least partially.
$CD14^+HLA\text{-}DR^{-/low}$ MDSC can be found in a great number of different cancers (see Table 1). They resemble monocytes in size and light scatter characteristics and express monocyte markers such as CD14 and S100A9.
However, we could show that they express even markers of more mature myeloid cells, namely CD80, CD83, and DC-Sign [107]. This subset has been described to exert suppression via ARG, iNOS, and suppressive cytokines.
As suppressive activity is a mandatory criterion for MDSC, some investigators have included expression of suppressive molecules, such as ARG1, into their phenotypic definition [94].
Unfortunately, many studies have described 'MDSC' without evaluating their ability to suppress T cells. Table 2 lists malignancies where MDSC have been described, though the lack of functional testing often precludes the conclusion that there really is an increased frequency of suppressive myeloid cells or that a certain treatment does or does not affect MDSC numbers [83, 86].

2.3.3.3 MDSC expansion

It is still not completely clear what the physiological role of MDSC is and how it is regulated. As for T_{regs}, that help to protect us from autoimmune disease, MDSC must have a natural function in regulating immune responses to self-antigens or infections. There is evidence from a number of infectious disease models, as well as from patients that suffered traumatic stress, that MDSC are recruited to dampen immune responses in order to prevent collateral damage to surrounding tissues [122-124].
Clearly, inflammatory pathways are at work during cancer development and progression. The overlap of these pathways with mechanisms regulating normal immune responses allows tumors to recruit MDSC that become pro-tumorigenic by suppressing anti-tumor T cell responses and contributing to the proinflammatory tumor microenvironment.
Many factors are implicated in inducing the exit of immature myeloid cells from the bone marrow. Several of these, including prostaglandin (PG)-E_2, stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-SCF), granulocyte-macrophage colony-stimulating

Table 2: *Human cancers in which MDSC have been described*

Cancer	Phenotype	T cell suppression tested	Suppressive Mechanism	Suspected/Excluded mechanisms	REF
adrenocortical cancer	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
appendiceal cancer	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$HLA\text{-}DR^-CD33^+CD11b^+CD15^+$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
B cell non-Hodgkin lymphoma	$CD14^+HLA\text{-}DR^{-/low}$*	YES	**ARG**		[110]
biliary tree adenocarcinoma	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
bladder cancer	$CD11b^+CD15^{high}CD33^{low}$ $CD11b^+CD15^{low}CD33^{high}$	YES	not tested	Treg induction	[118]
	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
	$SSC^{high}CD66b^+$	YES	not tested		[102]
breast cancer	$CD15^+$	YES	**ROS**		[92]
	$CD15^+CD11b^+CD33^+HLA\text{-}DR^-$	NO	not tested		[95]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$Lin^-HLA\text{-}DR^-CD33^+CD11b^{-/low}$	only *in vitro* MDSC tested	not tested		[88]
	$Lin^-DR^{-/low}$	YES	unknown	soluble factor, not NO	[80]
	$CD3^-HLA\text{-}DR^-CD33^+$	YES	not tested	STAT3 dependent	[116]
carcinoid tumor	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
cervical cancer	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
cholangiocarcinoma	$CD11b^+CD15^+CD33^+HLA\text{-}DR^-$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
colon cancer	$CD15^+$	YES	**ROS**		[92]
	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$CD3^-HLA\text{-}DR^-CD33^+$	YES	not tested	STAT3 dependent	[116]
	$CD15^+CD11b^+CD33^+HLA\text{-}DR^-$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
	$CD14^+$, PMN ($CD15^+$)	YES	not tested		[117]
colorectal carcinoma	$Lin^-HLA\text{-}DR^-CD33^+$ $CD11b^{-/low}$	only *in vitro* MDSC tested	not tested		[88]
esophageal cancer	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$Lin^-HLA\text{-}DR^-CD33^+CD11b^+$	NO	not tested	ARG	[89]
	$HLA\text{-}DR^-CD33^+CD11b^+CD15^+$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
fibrosarcoma	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
gall bladder cancer	$HLA\text{-}DR^-CD33^+CD11b^+CD15^+$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
gastric cancer	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$Lin^-HLA\text{-}DR^-CD33^+CD11b^+$	NO	not tested	ARG	[89]
	$HLA\text{-}DR^-CD33^+CD11b^+CD15^+$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
glioblastoma	$CD14^+HLA\text{-}DR^{-/low}$	indirectly	not tested		[113]
glioma	$Lin^-HLA\text{-}DR^-CD33^+$	only *in vitro* MDSC tested	not tested	*in vitro* MDSC make IL-10 + TGF-β	[82]
	$HLA\text{-}DR^-CD33^+CD15^+$ $CD33^+HLA\text{-}DR^-CD14^-CD15^-$ $CD33^+HLA\text{-}DR^-CD14^+$	YES	not tested	ARG	[119]
head and neck cancer	$Lin^-HLA\text{-}DR^-$	YES	unknown	soluble factor, not NO	[80]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$CD11b^+CD33^+CD14^-$	NO	not tested	ROS	[98]
	$CD11b^+CD33^+CD14^-$	YES (only tumor MDSC)	not tested		[97]
	$SSC^{high}CD66b^+$	YES	not tested		[102]
	$CD14^+$	YES	**ARG, iNOS**		[103]
hepatocellular carcinoma	$CD14^+HLA\text{-}DR^{-/low}$	YES	**ARG, T_{regs}**		[108]
	$CD14^+HLA\text{-}DR^{-/low}$	YES (NK cells)	unknown	NKp30-mediated	[109]
	$CD14^+HLA\text{-}DR^-CD33^+$ $HLA\text{-}DR^-CD33^+CD11b^+CD15^+$	NO	not tested		[120]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]

Table 2 continued

Cancer	Phenotype	T cell suppression tested	Suppressive Mechanism	suspected/excluded mechanisms	REF
leiomyosarcoma	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
melanoma	$CD14^+$, PMN ($CD15^+$)	YES	not tested		[117]
	$CD14^+HLA\text{-}DR^{-/low}$	only *in vitro* MDSC tested	**TGF-β**		[106]
	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
	$CD14^+HLA\text{-}DR^{-/low}$	YES	**TGF-β**	not ARG, not iNOS	[105]
	$CD14^+HLA\text{-}DR^{-/low}$, $CD11b^+$	YES	not tested		[114]
	$CD14^+HLA\text{-}DR^{-/low}$*	YES	**ARG**	ROS/STAT3 dependent	[107]
	$Lin^-HLA\text{-}DR^-CD33^+$	NO	not tested		[83]
	$Lin^-HLA\text{-}DR^-CD33^+$	NO	not tested		[84]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
mesothelioma	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
multiple myeloma	$CD14^+$	YES	**ARG, iNOS**		[103]
	$CD14^+HLA\text{-}DR^{-/low}$	NO	not tested		[112]
lung cancer (non-small cell)	$Lin^-HLA\text{-}DR^-$	YES	unknown	soluble factor, not NO	[80]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$CD11b^+CD33^+CD14^-CD15^+$	YES (ζ-chain)	**ARG, iNOS**		[100]
	$CD11b^+CD33^+(CD15^+)$	YES	not tested		[96]
	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
lung cancer (small cell)	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
osteosarcoma	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
ovarian cancer (ascites)	$CD14^+HLA\text{-}DR^{-/low}$	YES	**IL-10**		[104]
ovarian cancer	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
pancreatic cancer	$CD15^+$	YES	**ROS**		[92]
	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$Lin^-HLA\text{-}DR^-CD33^+CD11b^+$	NO	not tested	ARG	[89]
	$Lin^-HLA\text{-}DR^-CD33^+$ $CD11b^+CD33^+CD14^-$	YES	not tested		[90]
	$CD11b^+CD15^+CD33^+HLA\text{-}DR^-$ $CD14^+HLA\text{-}DR^-CD33^+$	NO	not tested		[120]
	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
prostate cancer	$CD14^+HLA\text{-}DR^{-/low}$	YES	not tested	IL-10	[111]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
	$CD3^-HLA\text{-}DR^-CD33^+$	YES	not tested	STAT3 dependent	[116]
rectal adenocarcinoma	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
renal cell carcinoma	$CD11b^+CD14^-$ $CD14^-CD66b^+$	YES	**ARG**		[101]
	$CD11b^+CD14^-CD15^+$	YES	not tested	Arg	[94]
	$CD15^+CD14^-$ $Lin^-HLA\text{-}DR^-CD33^+$	YES	**ARG**	trend for ROS	[93]
	$Lin^-HLA\text{-}DR^-CD33^+$	NO	not tested		[86]
	$Lin^-HLA\text{-}DR^-CD33^+$	YES	**ROS, NO**		[81]
	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
	$Lin^-HLA\text{-}DR^-CD33^+$	NO	not tested		[84]
	$Lin^-HLA\text{-}DR^-CD33^+$ $CD11b^+CD33^+CD14^-$	YES	not tested		[90]
	$Lin^-HLA\text{-}DR^-CD33^+$ $CD11b^+CD33^+CD14^-$	NO	not tested		[91]
	$CD11b^+CD33^+HLA\text{-}DR^-$	NO	not tested		[99]
	$CD14^+HLA\text{-}DR^{-/low}$*	NO	not tested		[115]
sarcoma	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
soft tissue sarcoma	$Lin^-HLA\text{-}DR^-CD33^+CD11b^+$ $CD11b^+CD33^+CD14^-$	YES	not tested		[90]
squamous cell carcinoma	$CD14^+HLA\text{-}DR^+CD33^+$ $CD14^-HLA\text{-}DR^-CD33^+$	YES	not tested		[121]
thymoma	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
thyroid cancer	$Lin^-HLA\text{-}DR^-CD33^+$	YES	not tested		[85]
	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]
urothelial cancer	$SSC^{high}CD66b^+$	YES	not tested		[102]
unknown primary	$Lin^-HLA\text{-}DR^-CD33^+(CD11b^+)$	YES	not tested		[87]

* No difference in frequency of $Lin^-HLA\text{-}DR^-CD33^+$

factor (GM-CSF), IL-6, and VEGF, can be produced by tumors or their stroma. Normally, bone marrow-derived immature myeloid cells will distribute throughout the body and differentiate into macrophages, granulocytes, or DC. Instead, the presence of (tumor-derived) inflammatory mediators arrests MDSC in their immature state.

In humans, it is difficult to assess which factors drive MDSC expansion. Mundy-Bosse *et al*. have described that plasma IL-6 correlates with the presence of granulocytic MDSC ($CD11b^+CD15^+CD33^+HLA\text{-}DR^-$), and plasma IL-10 levels with circulating $CD15^-$ MDSC [120]. Gabitass *et al.* reported Th2 skewing in cancer patients and a correlation of plasma IL-13 with $Lin^-HLA\text{-}DR^-CD33^+CD11b^+$ MDSC [89]. On the other hand, elevated plasma VEGF does not seem to be the mechanism behind increased $Lin^-HLA\text{-}DR^-CD33^+$ MDSC in glioma patients or $CD14^+HLA\text{-}DR^{-/low}$ MDSC in non-Hodgkin lymphoma [82, 110].

Recent studies, attempting to induce MDSC *in vitro* from PBMC or bone marrow aspirates, suggest that GM-CSF alone or in combination with G-CSF, IL-6, IL-1β, VEGF, or TNFα is sufficient to induce cells with MDSC phenotype and suppressive function [88, 125-128].

GM-CSF is frequently used as a vaccine adjuvant, though its role as an MDSC-inducing factor has raised the question whether its addition really benefits the vaccine-induced immune response. Filipazzi *et al.* observed an increase in MDCS after vaccinating melanoma patients with a GM-CSF-containing vaccine, and patients with higher MDSC levels tended to fall into the group of immunological non-responders [105]. Parmiani *et al*. compared clinical trials and animal studies where GM-CSF was used as an adjuvant and found that low-dose GM-CSF indeed had immunostimulatory properties, while higher doses abrogated vaccine success [129], probably due to expansion of MDSC.

2.3.3.4 MDSC activation

Though even immature myeloid cells that are not MDSC can exert suppressive function, these populations are not synonymous. MDSC are impaired in their ability to differentiate as long as they are exposed to tumor-derived factors and develop strong suppressive function as a result of activation. Activation is mediated by cytokines that are naturally produced by activated immune cells, for example IFN-γ, which is secreted by activated T and NK cells, IL-4, produced by Th2 cells, and IL-13, an immunoregulatory factor secreted by Th2 cells and NKT cells. Other MDSC-activating molecules include IL-1β, PG-E_2, and **toll-like receptor** (TLR) ligands.

Toll-like receptors (TLRs) are a family of pattern recognition receptors expressed by cells of the innate immune system. TLRs sense the presence of microbes inside or outside the cell and induce cell activation. TLR ligands include for example bacterial lipopolysaccharide (LPS), bacterial or viral DNA, and RNA.

Solito *et al.* have shown that strongly activated T cells are more easily suppressed by MDSC than those that receive weak stimulation, suggesting that factors derived from strongly stimulated T cells contribute to MDSC activation [88]. The same group even showed that the presence of activated T cells was necessary for *in vitro* generated MDSC to proliferate and maintain

their immature and suppressive phenotype, while co-culture with resting T cells resulted in myeloid cell differentiation [88]. This is in accordance with the observation that treatment with IL-2, a T cell-activating cytokine, has been reported to increase MDSC frequencies [84, 86, 101] and serum ARG1 levels [101] in renal cell carcinoma patients.
It should be noted that MDSC-recruiting factors, such as IL-1β and PG-E_2, also promote MDSC suppressiveness, so they can stimulate MDSC expansion as well as activation.
Importantly, MDSC can themselves produce proinflammatory stimuli such as IL-6, PG-E_2, and VEGF, thereby providing a positive feedback loop of MDSC recruitment [72, 130].

2.3.3.5 Suppressive mechanisms

MDSC can employ a wide range of suppressive mechanisms. Their 'weapon of choice' can depend on their monocytic or granulocytic subtype and often includes more than a single mechanism. Figure 3 provides an overview over the suppressive mechanisms that have been described in human MDSC.
Unfortunately, many studies do not formally test the suppressive pathway at work (see Tables 1 and 2), while others are satisfied in identifying one suppressive mediator where there might be several. With regard to therapeutic targeting of MDSC in different diseases, it would be interesting to test the full panel of candidate suppressive mechanisms in each described MDSC population and report even those that are irrelevant together with those that act alone, in parallel, or in synergy.

2.3.3.5.1 ARG1 and iNOS

Production of the enzyme ARG1 is one of the most frequently reported mechanisms used by MDSC to suppress T cells. ARG1 catabolizes the reaction from the amino acid L-arginine (L-Arg) to ornithine and urea. There are two ARG isoforms: ARG1 is a cytosolic enzyme constitutively expressed in the liver that can be induced in cells of the myeloid lineage, while ARG2 is found in the mitochondria of cells in the kidneys as well as the brain, small intestine, mammary glands, and macrophages [131]. In human blood, neutrophils are the main ARG1-producing population [132]. Different from mouse ARG1, which remains cytoplasmic, human ARG1 is exported from the cell in azurophil and/or gelatinase granules [132, 133]. High ARG1 activity and low L-Arg levels can therefore frequently be detected in serum of cancer patients [94, 101, 134, 135, Poschke, unpublished].
T cells are dependent on importing L-Arg from the microenvironment via cationic amino acid transporters (CAT) [131]. L-Arg deprivation leads to cell cycle arrest and prevents protein translation via the amino acid deficiency sensor general control non-repressible 2 (GCN2) pathway. ARG1-mediated defects in T cell function are described in greater detail in chapter 2.3.4.1.
Inducible nitric oxide synthase (iNOS), one of three NOS isoenzymes, is another L-Arg-catabolizing enzyme. As a byproduct of the conversion of L-Arg to citrullin by iNOS, nitric oxide (NO) is released.

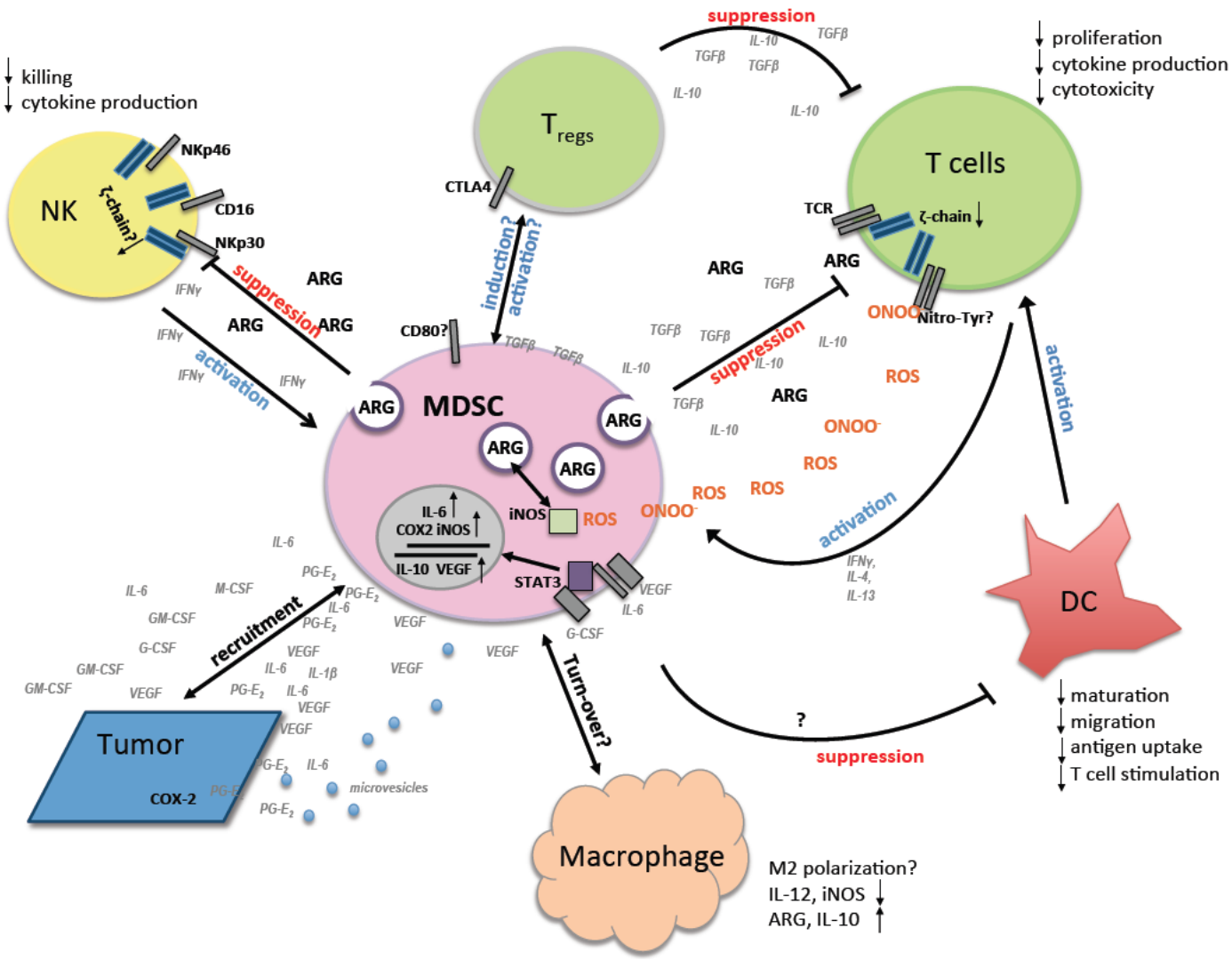


Figure 3: *Model of MDSC function in cancer patients. Mechanisms that are likely to be relevant, but have not yet been experimentally confirmed in humans, and interactions where the mechanism remains currently unknown are indicated by question-marks.*

Most molecules/receptors on the surface of immune cells are randomly distributed across the cell membrane, though some of them can be clustered together. When a lymphocyte and an APC meet they start to re-arrange molecules relevant for antigen presentation, recognition, and co-stimulation, so that most of them end up in small areas of the cell membranes that are facing each other, the **immunological synapse**. This re-arrangement makes receptor-ligand interactions more likely and allows longer contacts, thereby amplifying the exchanged signals. Secreted molecules will likely give signals only within the immunological synapse and not affect surrounding, uninvolved cells.

NO can act as an intracellular messenger, but is converted to the radical peroxynitrite ($ONOO^-$) in presence of ROS. Secretion of reactive nitrogen species can cause protein nitration, most frequently affecting tyrosine side chains. Nagaraj *et al.* have shown that nitrotyrosine accumulation in the T cell receptor impaired its ability to interact with peptide-MHC complexes and therefore prevented T cell responses in a mouse model [136]. Of course the TCR will not be exclusively affected by NO release from MDSC. Though this has not been studied yet, one can expect all proteins in close proximity to the NO source, for example those residing in the **immunological synapse**, to be affected by protein nitration. The observed immunosuppression could therefore be mediated by TCR dysfunction in combination with a variety of other mechanisms, such as lack of co-stimulation. Increased protein nitration has been observed in human cancers, though it was not confirmed whether this was mediated by MDSC [137, 138]. Recent data by Molon *et al.* suggest that reactive nitrogen species might even act over longer distances because nitration of chemokines in the tumor microenvironment hampers T cell recruitment into the tumor [139].
Normally, ARG and iNOS are not expressed by the same cell. For example M1- and M2-polarized macrophages are characterized by expression of iNOS and ARG1, respectively (see chapter 2.3.3.9). This is probably due to the fact that ARG1 and iNOS regulate each other. Particularly ARG1-mediated L-Arg consumption prevents iNOS expression [140, 141]. The ability to co-express ARG1 and iNOS seems to be a hallmark of MDSC and affects the suppression they exert. When iNOS is expressed under conditions of high L-Arg consumption, e.g., by co-expression of ARG1, the catabolized reaction is slightly modified and now yields ROS as a side product in addition to NO [142]. For more information see even Figure 4 on page 32.

2.3.3.5.2 Oxidative stress

As discussed above, ROS production by MDSC can be a consequence of L-Arg metabolism. An alternative and probably more dominant mechanism of ROS production involves the family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. NADPH oxidases, of which NOX2 is the most important isoform in human neutrophils and macrophages, are multi-protein complexes that release ROS into the extracellular space upon activation [143]. NADPH oxidase expression in neutrophils is crucial to control infections, as patients with chronic granulomatous disease, who have a mutation in this multi-subunit enzyme, suffer from frequent and severe infections [144]. Corzo *et al.* showed that MDSC derived from head and neck cancer patients produced

more ROS than equivalent cells from healthy controls [98]. In a mouse model these investigators further showed that NADPH oxidase was overexpressed in a signal transducer and activator of transcription (STAT)3-dependent manner in immature myeloid cells from tumor-bearing compared with tumor-free mice [98]. **Knock-down** of the important NADPH oxidase subunit $gp91^{phox}$ prevented suppressive activity of murine MDSC. This source of ROS in MDSC has not been thoroughly investigated in humans. Similar to Corzo *et al.,* we observed increased ROS production in melanoma patient-derived MDSC and a connection between levels of oxidative stress in myeloid cells and presence of phosphorylated STAT3 (Paper I) [107]. These findings suggest that ROS production in human MDSC is also regulated in a STAT3-dependent way. However, mRNA levels of the NADPH oxidase subunits $gp91^{phox}$ or $p47^{phox}$ were similar in $CD14^{+}HLA\text{-}DR^{-/low}$ cells from melanoma patients and controls (Paper I) [107], suggesting regulation on a different level or ROS production through an alternative pathway.

Knock-down indicates that a gene or gene product is transiently shut off in a cell. This can be achieved by molecular biology techniques or by genetically introducing an inducible silencer close to the gene of interest. This technique is used to understand the role of a specific molecule in a cell by testing what happens when it is no longer there. Gene knock-down is usually denoted as for example $gp91^{-/-}$, indicating that both copies of the gp91 gene should be silenced.

Interestingly, Lechner *et al*. found up-regulation of NADPH oxidase in $Lin^{-}CD33^{+}HLA\text{-}DR^{low}$ MDSC that were induced *in vitro* by cytokines or tumor cell co-culture [125, 126].

2.3.3.5.3 Cytokines

Besides overexpression of ARG1 and ROS production, the most important suppressive mechanisms of human MDSC seem to involve suppressive cytokines. In 1999, Loercher *et al.* published one of the first descriptions of MDSC in humans. They found that monocytes in ovarian cancer ascites expressed low HLA-DR and could suppress T cell responses via secretion of IL-10 in combination with TGF-β [104]. Similarly, Vuk-Pavlovic *et al.* showed that $CD14^{+}HLA\text{-}DR^{-/low}$ MDSC from prostate cancer patients produced more IL-10 than their $HLA\text{-}DR^{+}$ counterparts, though they did not formally test whether this was the mechanism underlying the T cell suppression they observed [111].
TGF-β contributed to suppression exerted by MDSC generated *in vitro* [125, 126] or by $CD14^{+}HLA\text{-}DR^{-/low}$ MDSC isolated from melanoma patients by L. Rivoltini's group [105, 106]. However, $CD14^{+}HLA\text{-}DR^{-/low}$ MDSC isolated from melanoma patients by our laboratory (Paper I) [107] or from hepatocellular carcinoma patients by Hoechst *et al*. [108], as well as monocytic and granulocytic MDSC in bladder cancer patients [118], did not suppress via this mechanism. Interestingly, Hoechst *et al.* later showed that membrane-bound TGF-β on MDSC indirectly contributes to suppression by inducing T_{regs} [108].

2.3.3.5.4 Collaboration with T_{regs}

We did not find a correlation between natural T_{regs} and MDSC in the blood of melanoma patients (Paper I) [107], but this has been observed by others in renal cell carcinoma [93], glioblastoma [113], pancreas, esophageal and gastric cancer [89]. Several studies have also shown that isolated and *in vitro* generated MDSC can induce the expansion of regulatory T cells in MDSC-T cell co-cultures [108, 118, 125, 145]. Hoechst *et al.* recently showed that $CD14^+HLA\text{-}DR^-$ cells convert conventional T cells into $FoxP3^+$ and IL-10-producing T cells with suppressive function, while $CD14^+HLA\text{-}DR^+$ monocytes instead promote conversion into Th17 cells [145].
These studies indicate that MDSC and T_{regs} can collaborate, and that T_{regs} can contribute to the suppressive activity observed as a result of MDSC expansion. Interestingly, there is one study showing that, under certain conditions, even the opposite can be the case: in a tumor model including long-term T_{reg} depletion, MDSC were gradually induced due to lipoxin A_4 production and could accelerate tumor progression [63].
Several other studies have addressed the collaboration of MDSC and T_{regs} in mouse tumor models. MDSC can take up, process, and present antigen to T_{regs} [75, 146]. T_{reg} induction was dependent on ARG1 but not TGF-β in a preclinical model of B cell lymphoma [146] and required IFN-γ and IL-10, but not iNOS, in a colon cancer model [75]. Pan *et al.* showed that blocking of the murine SCF receptor on MDSC restored T cell proliferation and reduced T_{reg} expansion [78], and Yang *et al*. reported that CD80 expression on MDSC could induce T_{reg} expansion via CTLA-4 stimulation [147].

2.3.3.5.5 Suppressive mechanisms so far reported only in mouse models

Cysteine sequestration

T cells are unable to generate the amino acid cysteine, and, unlike many other cells, cannot import cystine, the oxidized form of cysteine, because they lack the x_c^- transporter. This makes T cells dependent on cysteine provided by antigen-presenting cells. Due to the close proximity during antigen presentation, cysteine can be directly transferred between the neutral amino acid transporters (ASC) of APC and T cells or maintained in its reduced form by APC-derived reducing agents such as thioredoxin until taken up by the T cell.
Murine MDSC express the x_c^- transporter, but not ASC, allowing them to import and sequester cysteine within the cell. Depletion of the cystine pool requires APC to synthesize their own cysteine and limits the amount available for them to export, thereby depriving T cells of this essential amino acid [96].
In addition to its role in protein synthesis, cysteine is also a substrate for the production of glutathione, a redox buffer molecule. Consequently, conditions of low cysteine availability will also make cells more sensitive to oxidative stress [148]. Feeding mice with N-acetyl cysteine, a form of cysteine that cannot be oxidized in the extracellular milieu, alleviated MSDC-mediated suppression and arrested tumor growth [149, 150].

In a comparison of mRNA levels encoding amino acid transporters in $CD14^{+}HLA\text{-}DR^{-/low}$ cells from melanoma patients and healthy controls, we could not detect significant differences between these two populations [Poschke and Mougiakakos, unpublished], so the importance of cysteine sequestration in human MDSC still remains an open question.

Impairment of T cell homing

MDSC constitutively express active ADAM 17, a protease that cleaves the homing receptor CD62L. CD62L is normally expressed on naive T cells and allows them to enter lymphatic tissues, where they can sample antigens presented by APC. If CD62L is cleaved off by MDSC-expressed ADAM 17, T cell trafficking is impaired and their priming by activated APC in the lymph nodes is prevented [151].

CD80

In a mouse model of ovarian cancer, Yang *et al.* showed that tumor-induced, but not naïve, $CD11b^{+}Gr1^{+}$ cells expressed CD80 [119]. This molecule was crucial for their suppressive activity, as $CD80^{-/-}$ MDSC exhibited reduced suppression. *In vivo* treatment with a CD80-blocking antibody significantly delayed tumor growth in this model. MDSC suppression was indirect via stimulation of regulatory T cells expressing the CD80 ligand CTLA-4 [119]. The same group later showed that tumor-bearing CD80-deficient mice exhibited reduced ARG1 expression and activity in their MDSC [152].
Of note, we also found overexpression of CD80 in melanoma MDSC as compared with $CD14^{+}HLA\text{-}DR^{-/low}$ cells from healthy donors, though we could not confirm that this molecule contributed to MDSC-mediated T cell suppression (Paper I) [107].

Pro-angiogenic activity

Due to their ability to secrete VEGF, MDSC have been implicated in promoting tumor angiogenesis. Interestingly, Yang *et al.* recently revealed a more direct role of MDSC in tumor vascularization by showing that MDSC co-injected with tumor cells lined the endothelial walls of newly formed tumor vasculature and could differentiate into cells with endothelial cell characteristics [153].
The same group also showed that MDSC from tumor-bearing mice produced high levels of matrix metallopeptidase (MMP) 9, which favored their ability to promote tumorigenesis and angiogenesis [153]. MMP9 can enzymatically digest the extracellular matrix and has therefore been implicated in promoting metastasis, but also in increasing the bioavailability of VEGF [154, 155].
These striking findings reveal a new level of MDSC plasticity and suggest a mechanism by which MDSC can promote metastasis that awaits confirmation in human cancer.

2.3.3.6 Suppression of NK cells

Little is known about the suppression of NK cells by human MDSC. Hoechst *et al.* demonstrated that $CD14^{+}HLA\text{-}DR^{-/low}$, but not $CD14^{+}HLA\text{-}DR^{+}$ cells, potently inhibited NK cell proliferation, cytokine production, and cytotoxicity [109]. This

was not mediated by ARG, iNOS, or IDO, but required MDSC-NK interactions involving the NK cell activating receptor NKp30 [109].
In the mouse, Liu *et al*. found an inverse correlation of NK cell activation and MDSC frequency and showed that MDSC from tumor-bearing mice could inhibit NK cytotoxicity in a cell-cell contact-dependent way by blocking perforin expression in the NK cells [156]. Furthermore, Li *et al*. showed that MDSC could inhibit NK cytotoxicity, NKG2D expression, and IFN-γ production both *in vitro* and *in vivo*. Suppression was mediated by membrane-bound TGF-β, and the function of hepatic NK cells in a liver cancer model could be restored by MDSC depletion [157].
Interestingly, and in contrast to the studies described above, Nausch *et al*. showed that MDSC did not suppress, but instead activated NK cells in a mouse model of lymphoma. NK cells were induced to produce IFN-γ via stimulation of their activating NKG2D receptor by Rae-1 expressed on MDSC [158].

2.3.3.7 Role of STATs in MDSC recruitment, activation, and function

Many MDSC-recruiting signals, including for example G-CSF, VEGF, and IL-6, converge in the signal-transducing molecule STAT3. Importantly, STAT3 signaling controls a number of molecules important for MDSC recruitment and suppressiveness, such as iNOS, VEGF, cyclooxygenase (COX)-2, IL-6, and IL-10, thus providing a positive feedback loop [159].
STAT3 signaling prevents differentiation of MDSC, as pharmacological inhibition or knock-down of STAT3 facilitates the conversion of MDSC to differentiated cells without suppressive activity [98, 160, 161]. In mouse MDSC, this blockade of differentiation seems to be mediated by the transcription factor CCAAT-enhancer-binding protein beta (C/EBPβ), which has also been suggested as an MDSC-defining marker [127].
We were first to confirm an important role of STAT3 in human MDSC, showing that it was overexpressed, present in its active form and associated with ROS production, and that its inhibition completely abrogated the suppressive activity of melanoma MDSC (Paper I) [107].
As they are central signal-transducing molecules, even other STAT molecules have been shown to be important in (murine) MDSC. STAT1 plays a dominant role in activation and suppression exerted by (monocytic) MDSC, as it transduces IL-1β and IFN-γ signals into up-regulation of ARG1 and iNOS [162]. Further, STAT5 is an important transducer of GM-CSF signals involved in MDSC recruitment and STAT6 is downstream of IL-4Rα and can regulate T cell suppression by up-regulating ARG1 and TGF-β [142, 163].

2.3.3.8 Impaired differentiation

As mentioned earlier, MDSC contain immature cells that are inhibited in their differentiation along the myeloid lineage due to tumor-derived factors or autocrine feedback through factors that they produce.
Studies in mouse models suggest that particularly STAT3 signaling and ROS production seem to be important to keep MDSC arrested in an immature state. The reduced frequency of mature DC in the blood of cancer patients [83, 85,

112] could be due to impaired myeloid differentiation, as suggested by a number of *in vitro* studies:
Almand *et al.* could differentiate Lin^-DR^- myeloid cells into DC but not into granulocytes. Only a minority of cells seemed capable of differentiation, but the combination of GM-CSF with all-*trans* retinoic acid (ATRA) improved DC yield and stimulatory capacity [80]. Ko *et al*. did observe up-regulation of DC markers such as CD80 and CD40 after culture of $Lin^-CD33^+HLA\text{-}DR^-$ cells with IL-4 and GM-CSF, though this was not compared with, e.g., a $HLA\text{-}DR^+$ control population [93]. Hoechst *et al.* found that, while $CD14^+HLA\text{-}DR^+$ cells efficiently differentiated to immature and mature DC in presence of IL-4, GM-CSF, and bacterial lipopolysaccaride (LPS), $CD14^+HLA\text{-}DR^{-/low}$ cells retained high CD14 and did not acquire DC characteristics [108]. Interestingly, Vuk-Pavlovic *et al*. found that, while there was no difference in DC yield, the inability of $CD14^+$ cells isolated from prostate cancer patients to differentiate into fully mature DC correlated with the percentage of $HLA\text{-}DR^-$ cells in the culture [111]. Further, we showed that, when high frequencies of $HLA\text{-}DR^-$ cells were present in monocyte cultures, the resulting DC were less mature, impaired in their ability to take up antigen, migrate, and induce T cell proliferation. This was partially due to the deficient differentiation of $HLA\text{-}DR^-$ cells, but also to a negative effect of this population on $HLA\text{-}DR^+$ cells in the same culture (Paper II).
Clearly, their suppressive activity should not be the sole concern when thinking of MDSC. The inability of cancer-recruited immature myeloid cells to differentiate into mature stimulatory cells will interfere with the priming and expansion of potentially tumor-reactive cells, and their secreted factors can aid the immunosuppressive milieu and promote Th2 skewing.

2.3.3.9 Relationship of MDSC and macrophages

Macrophages are mature myeloid cells that can be found in many tissues and that partially overlap with MDSC in phenotype, recruitment/activation, and effector mechanisms. The relationship between MDSC and macrophages is still not completely understood, especially in humans, where access to tissues is difficult, and both MDSC and macrophages are hard to define by single unique markers.
Depending on the signals they receive, macrophages become either classically or alternatively activated, M1 or M2 macrophages, respectively. M1 macrophages have antimicrobial and anti-tumor activity and produce high levels of ROS and reactive nitrogen species, as well as IL-12, while M2 macrophages can express IL-10 and, in the mouse, ARG1, and are considered to be pro-angiogenic, pro-tumorigenic and immunosuppressive. Of note, the cytokines inducing macrophage polarization, including IFN-γ for M1 and IL-4 and IL-13 for M2 macrophages, overlap with those leading to MDSC recruitment and activation. In cancer, tumor-associated macrophages (TAM) are frequently observed and considered to be a tumor-induced type of M2-polarized macrophages. Due to the overlap in surface markers and function, MDSC and M2 macrophages are sometimes not clearly distinguished from each other, especially in older publications. Some evidence suggests that

MDSC can differentiate into TAM, explaining why these two populations share so many characteristics.
Eruslanov *et al.* have shown that bone marrow cells exposed to tumor-conditioned medium become enriched for MDSC and M2 macrophages [164]. This was mediated via increased PG-E_2 production in the myeloid cells, though it was not investigated which cells were producing PG-E_2 and whether one of the myeloid populations was controlling the induction of the other [164]. Kusmartsev *et al.* showed that, after adoptive transfer of $Gr1^+$ cells into tumor-bearing mice, the majority of donor cells recovered from the tumor three days later were TAM that could suppress T cells in ARG1- and NO-dependent ways [165]. Corzo *et al.* recently demonstrated that hypoxia in the tumor induced differentiation of MDSC into TAM that could suppress T cells in an antigen-independent way. The shift in phenotype was associated with a loss of ROS production and increased ARG1 and iNOS expression mediated by hypoxia inducible transcription factor (HIF)-1α [166].
On the other hand, *in vitro* studies by Sinha *et al*. could show that there also is cross-talk between MDSC and macrophages. MDSC-macrophage co-cultures induced IL-10 production by MDSC and reduced IL-12 production by macrophages in a cell-cell contact-dependent way [167].

2.3.3.10 MDSC and survival in cancer patients

It seems clear that MDSC can pose a significant hurdle to anti-tumor immunity. At the same time, the extent to which the immune system really controls tumor growth is still debated. In favor of a role for tumor immunosurveillance, several recent papers have identified increased MDSC levels as predictors of poor prognosis. Mundy-Bosse *et al*. reported that in a mixed populations of cancer patients, the presence of $CD14^-CD33^+HLA\text{-}DR^-$ MDSC correlated with reduced survival, while, somewhat surprisingly, $CD14^+HLA\text{-}DR^-$ cells were associated with a better survival [120]. Studies by Gabitass *et al.* and Solito *et al.* reported that $Lin^-CD33^+HLA\text{-}DR^{-/low}$ cells predicted poor overall survival in pancreatic, esophageal, gastric [89], as well as breast and colon cancer patients [88].
The finding that presence of MDSC can represent an independent prognostic factor in a number of different malignancies makes them an attractive and promising therapeutic target. MDSC-targeting strategies are discussed in section 2.3.3.12.

2.3.3.11 MDSC in other conditions

Much attention has focused on the role of MDSC in cancer. As of yet, very few studies have shown a role for MDSC in other human diseases: $CD11b^+$ cells isolated after influenza virus infection inhibited MLRs by utilizing ARG and iNOS [128], and patients with inflammatory bowel disease have increased numbers of $CD14^+HLA\text{-}DR^-$ MDSC that suppress T cell proliferation and IFN-γ production [168]. Very recently, increased levels of ARG1 expressing $CD11b^+MHC\text{-}II^-$ myeloid cells were detected in patients with pulmonary hypertension, an inflammatory lung condition [169]. Though the evidence is still

sparse, these findings indicate a role for MDSC in both infection and (autoimmune) inflammation.

Studies performed in mouse models further confirm this notion: MDSC were found to be expanded in bone marrow, spleen, and lymph nodes during sepsis [170, 171], and in the spleen after burn injury [172], traumatic injury [173], and many types of infections [123, 174, 175].

Under conditions of sepsis and trauma, an alternative function of MDSC deserves consideration. As they are efficient producers of ROS, MDSC could also exert antimicrobial function and thus protect the host from opportunistic infection while dealing with other stress. Interestingly, MDSC isolated from mice during sepsis or after burn injury also produce proinflammatory mediators such as TNF-α, macrophage inflammatory protein (MIP)1-α, monocyte chemotactic protein (MCP)-1, and 'regulated upon activation, normal T-cell expressed, and secreted' (RANTES), that have not been observed in tumor-induced MDSC [176, 177]. Mice deficient in IL-6 signaling succumb to sepsis and can be rescued by adoptive transfer of $CD11b^+Gr1^+$ cells, suggesting that MDSC exert a protective role in this setting [171].

Increased myelopoiesis frequently occurs during parasite infection, and the accumulation of $CD11b^+Gr1^+$ cells has been described as a consequence of infection with several different parasite species [123]. This increase in potential MDSC could be a result of increased levels of GM-CSF, M-CSF, IL-3, IL-6, IL-13, TLR stimulation, and IFN-γ production induced by parasite infections, especially in the acute phase. T cell suppressive activity of the accumulated myeloid cells was confirmed in several preclinical models [178-180]. Interestingly, Pereira *et al.* recently demonstrated that NO-mediated killing of parasites by MDSC helps to control Leishmania major infections in mice despite concurrent T cell suppression [181]. Of note, some parasite-derived factors can exhibit a direct inhibitory effect on myeloid differentiation [182] and trigger expansion of $CD11b^+Gr1^+$ cells in the spleen [183].

These reports suggest that, in contrast to cancer where MDSC predominantly contribute to immune escape, suppressive myeloid cells can be a 'double-edged sword' during infection: they might favor microbe survival and establishment of chronic infections, but also prevent tissue damage, immune-related morbidity and even eradicate the pathogen in some cases.

Accumulation of myeloid cells has also been observed in a number of animal models of autoimmune diseases [184-188]. Isolated myeloid cells displayed characteristics of MDSC and could suppress T cell activity, though their depletion did not affect disease outcome in most cases [189].

A mouse model of IBD showed that MDSC could induce tolerance after repeated immune stimulation or early on, when given as an adoptive transfer together with the first autoreactive T cell infusion [168].

Somewhat surprisingly, MDSC were found to contribute to disease severity in experimental autoimmune encephalomyelitis, a model for the autoimmune disease multiple sclerosis [190]. The disease-supporting role of MDSC in this case could be due to their differentiation into mature DC and macrophages after reaching the central nervous system [190].

2.3.3.12 Therapeutic targeting of MDSC

Since MDSC are important hurdles for naturally occurring or therapeutically induced anti-tumor immunity, their therapeutic targeting is highly attractive. Unfortunately, a specific depletion is impossible due to the lack of unique markers. Therapies tested so far have therefore included a number of rather unspecific approaches. For example, the chemotherapeutic drug gemcitabine has been shown to induce MDSC apoptosis in preclinical models and has been successfully applied alone or in combination with other therapies, including immunotherapies, though the effect of gemcitabine on MDSC in cancer patients remains to be studied. It should be noted that the chemotherapeutic drug cyclophosphamide, which is often used in conjunction with immunotherapy, has been shown to increase MDSC frequencies [87].

2.3.3.12.1 Differentiation

As MDSC are immature cells, differentiating them could not only relieve suppression, but even increase the number of mature and possibly anti-tumorigenic myeloid cells, such as DC or M1-polarized macrophages. As discussed above, MDSC can be differentiated *in vitro* with varying success using standard DC generation protocols.

- A stronger differentiation stimulus can be provided by all-*trans* retinoic acid (ATRA), a vitamin A metabolite that is used clinically for the treatment of acute promyelocytic leukemia [191]. MDSC express high levels of the ATRA receptor RAR-γ [81] and can be induced to differentiate by ATRA via up-regulation of glutathione synthase and reduced ROS production [91]. Administration of ATRA to renal cell carcinoma patients reduced the number of circulating immature myeloid cells in those patients where high serum ATRA levels could be achieved. This was associated with an improved ability of mononuclear cells to stimulate proliferation of allogeneic T cells [86].
- Vitamin D_3 is another molecule known to induce myeloid differentiation. Lathers *et al.* showed that vitamin D_3 administration reduced the frequency of circulating $CD34^+$ cells, increased HLA-DR levels, as well as plasma IL-12 and IFN-γ levels and T cell responses in patients with head and neck cancer. Of note, plasma IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, and TGF-β levels remained unchanged [192].

2.3.3.12.2 Prevention of MDSC expansion

MDSC persistence seems to be tumor dependent, and tumor resection or curative treatment can normalize MDSC frequencies [87, 100], indicating that MDSC turn over and have to be replenished continuously. As a consequence, blocking MDSC recruitment could decrease MDSC accumulation over time, giving tumor-specific T cells a chance to act. This has been tried with a number of different molecules:

- The tyrosine kinase inhibitor sunitinib which blocks pathways upstream of STAT3, such as signaling through the receptors for VEGF, platelet-derived growth factor (PDGF), fms-like tyrosine kinase 3 (Flt3)-ligand, and SCF, reduced MDSC levels and improved T cell responses in metastatic renal cell

carcinoma [93]. Preliminary data from the same group even showed that neoadjuvant sunitinib treatment in some cases reduced MDSC infiltration in the tumor [99]. In a mixed group of cancer patients, van Cruijsen *et al.* noted a slight but non-significant decrease of MDSC after sunitinib treatment and increased frequencies of myeloid DC in the blood of responding patients [115].

- Cancer patients treated with VEGF-trap, a VEGF-binding protein, did not show significant differences in MDSC levels. However, increased DC frequencies and improved T cell responses were observed in those patients that did have lowered post-treatment MDSC levels [85]. Similarly, treatment of renal cell carcinoma patients with the anti-VEGF antibody bevacizumab did not reduce circulating MDSC levels, but instead increased MDSC frequencies and plasma ARG1 levels when given in combination with IL-2 [101].
- *In vitro* studies using mouse MDSC showed that a COX-2 inhibitor (LM-185) allowed myeloid differentiation even in the presence of tumor and reduced T cell suppression by MDSC [164]. Treatment of tumor-bearing mice with the COX-2 inhibitor SC58236 prevented MDSC accumulation *in vivo* and delayed tumor growth [167]. Talmadge *et al.* showed that the COX-2 inhibitor celecoxib delayed induction of chemically induced tumors, increased numbers of tumor-infiltrating T cells, reduced MDSC, and normalized mRNA levels of iNOS and ARG1 measured in the spleen [193].
- Exosomes have been shown to contribute to MDSC induction in cancer patients and *in vitro* [105, 116]. Amiloride, a drug used to treat high blood pressure, reduces exosome release. Serum from cancer patients treated with amiloride had an impaired ability to activate MDSC, and MDSC isolated from their blood had a reduced suppressive activity compared with MDSC isolated before treatment [116].

2.3.3.12.3 Preventing suppression

A third strategy to target MDSC is to inhibit their suppressive pathways. This is complicated due to the fact that suppressive mechanisms vary between MDSC subsets, and between different cancers, and that there can be more than one mechanism at work.

Of course, the prime targets of drugs targeting MDSC-mediated suppression have been ARG1 and iNOS. Nor-NOHA and L-NMMA, ARG1 and iNOS inhibitors frequently used *in vitro,* are not suitable for *in vivo* usage due to their side effects. Currently, several molecules that can target both enzymes simultaneously seem to be promising candidate drugs:

- Nitroaspririn is acetyl-salicylic acid with an additional NO-releasing group and was originally developed to reduce side effects of long-term aspirin intake. Work in preclinical models showed that nitroaspirin can exert direct inhibitory effects on both iNOS and ARG1, but also act indirectly by preventing up-regulation of these molecules. In addition, nitroaspirin acts as an antioxidant, thereby detoxifying the tumor microenvironment and preventing the ROS-mediated differentiation arrest of MDSC [128]. Interestingly, nitroaspirin alone has only a minor effect on tumor growth, but can act as a potent adjuvant when given in combination with immunotherapy [128].

• The phosphodiesterase 5 inhibitor sildenafil (sold as Viagra®) down-regulated both ARG1 and iNOS, as well as IL-4Rα, in mouse MDSC. It delayed tumor growth, and acted synergistically with adoptive T cell transfer in a mouse model of cancer. *In vitro* addition of sildenafil to cancer patients' PBMC could significantly improve T cell proliferation [103]. It was suggested that destabilization of iNOS mRNA is in part responsible for alleviating suppression, while ARG1 expression is probably inhibited due to interference with signaling from IL-4Rα [194]. Several clinical trials, for example using sildenafil to treat erectile dysfunction in prostate cancer patients or to prevent heart damage by doxorubicin treatment, are underway, and probably enrolling patients fast (http://www.cancer.gov/clinicaltrials). Hopefully, immunomonitoring in these trials will be performed to reveal whether sildenafil can be used to target MDSC *in vivo* in cancer patients.
• The synthetic triterpenoid CDDO-ME completely abrogated *in vitro* suppressive activity of MDSC isolated from renal cell carcinoma patients. A clinical trial combining CDDO-ME with gemcitabine was well tolerated but showed no difference in MDSC levels after treatment. Interestingly, T cell responses were significantly improved after a 2-week treatment period. In mouse models, CDDO-ME treatment significantly decreased tumor growth. Even in these models, MDSC frequencies remained constant during treatment, but suppressive activity by MDSC was abrogated due to decreased ROS production, while iNOS and ARG1 levels were unaffected [90].

2.3.3.13 Therapeutic application of MDSC

Considering the potent immune suppression that can be exerted by MDSC, these cells promise to be useful in treating conditions of overwhelming immune activation.
Dougast *et al.* found that rats tolerant to mismatched kidney transplants had high levels of MHC class II negative myeloid cells in their circulation as well as in the graft [195]. These cells suppressed T cell function in an iNOS-dependent fashion, and iNOS inhibition could induce graft rejection. Several other groups have shown that adoptive transfer of *in vivo* or *in vitro* induced MDSC could prevent allograft rejection in mice [196-198]. *In vitro* induced MDSC were also able to inhibit graft-versus-host disease by L-Arg-dependent mechanisms [199, 200].
It is surprising that MDSC that can suppress T cell responses *in vitro* accumulate in autoimmune disease and do not seem sufficiently able to suppress *in vivo* immune responses to inhibit the disease. However, one should keep in mind that adoptive transfer of MDSC has a beneficial effect on autoimmune pathology in some models [168, 187, 188]. This suggests that the infusion of 'fresh' MDSC unexposed to the inflammatory microenvironment might be capable of overcoming the mechanisms preventing their natural *in vivo* activity under conditions of autoimmune inflammation.
In conclusion, while MDSC are an obstacle to tumor immunology, they might be an asset in the treatment of autoimmune diseases and for the prevention of graft rejection after transplantation. When considering MDSC as a treatment

option one should take into account the associated risk that infusing large numbers of MDSC could result in general immune suppression and accelerate progression of undiagnosed tumors.

2.3.4 T cell dysfunction

T cell differentiation, activation, and function are complex processes and almost every involved molecule can be affected in cancer. In this chapter I will therefore only discuss concepts relevant to the papers included in this thesis.

2.3.4.1 Down-regulation of the ζ-chain

The ζ-chain is a 16-kD transmembrane protein that is expressed as a disulfide linked homodimer by T and NK cells. In its cytoplasmic domain, it contains three immunoreceptor tyrosin-based activation motifs (ITAMs). Phosphorylation of these motifs transduces activating signals that, via the transcription factors nuclear factor κB (NFκB) and nuclear factor of activated T cells (NFAT), result in proliferation and cytokine secretion. In T cells, the ζ-chain transduces signals from the TCR complex and in NK cells, it is associated with the activating receptors NKp30, NKp46, and CD16.
Down-regulation of the ζ-chain has been described in many different cancers [201-209] and often correlates not only with reduced lymphocyte function, but also with poor prognosis [210-213]. ζ-chain loss has also been observed in autoimmune diseases, during infection, or after traumatic injury [141, 214-217].
ζ-chain loss can also be detected after T cell stimulation, because TCR engagement results in internalization and degradation of the entire TCR complex. This renders the T cell temporarily unresponsive until new receptor complexes appear on the cell surface about 24 hours after initial stimulation, though levels are not normalized until about 72h after antigen encounter [218]. Of note, the mechanism of tumor-mediated ζ-chain loss appears to be a different one and depends on L-Arg starvation and exposure to oxidative stress [141, 214].
T cells with low levels of ζ-chain expression have also been reported to exhibit other defects, such as low expression of the CD3 γ-chain, TNF, granzyme, and LCK and FYN, two kinases involved in signal transduction from the TCR [219].
Work by Bronstein-Sitton *et al*. suggests that chronic antigen exposure could lead to loss of ζ-chain expression in mouse models. However, not the chronic TCR stimulation itself, but rather the inflammatory microenvironment, including accumulation of MDSC, seems to mediate the T cell dysfunction [220]. The same group further showed that disappearance of the ζ-chain is a result of lysosomal degradation [220], while others find that it is also mediated on the transcriptional level [221]. This discrepancy could be explained by differences between human and mouse T cells and different diseases studied.
ARG1 is an important mediator of T cell suppression by MDSC and has been shown to cause loss of T cell ζ-chain expression [94, 222].

The **cell cycle** is a tightly regulated process organizing cell survival and division. It can be divided into five phases and the transition between phases is only possible when a number of checkpoints is successfully passed. **G_0** phase is a resting phase occupied by non-dividing cells; during **G_1** phase, cells increase their size and check if they are prepared for DNA synthesis; during **S** phase (synthesis), the cellular DNA is replicated, reaching a double set of chromosomes in **G_2** phase. During M phase (mitosis), the cellular content is organized to be divided into two daughter cells.

T cells activated under low L-Arg conditions retain some functional capacity, such as the ability to produce IL-2 and up-regulate the activation marker CD69, but they fail to proliferate and instead become arrested in the G_0-G_1 phase of the **cell cycle**. *In vitro* studies with human T cells could show that absence of L-Arg prevented up-regulation of cyclin D3 and cdk4, and reduced Rb phosphorylation and levels of the E2F-1 transcription factor, which are all needed for the cell to progress through G_1 and into the S phase. Decreased levels of these proteins were due to reduced transcription, mRNA stability, and translation [222, 223]. The same group also showed that the lack of L-Arg activates general control non-repressed (GCN)-2 kinase, a sensor for the accumulation of empty tRNAs that can prevent initiation of translation during amino acid starvation. Consequently, T cells from GCN2$^{-/-}$ mice could be activated and proliferated in absence of L-Arg [223].

Even production of ROS by MDSC, granulocytes, or macrophages has been shown to induce ζ-chain loss in lymphocytes, and T cell function can be rescued by addition of ROS-scavenging molecules such as catalase [92, 224]. Though it has been suggested by some [225-227], many investigators did not find a correlation between the loss of ζ-chain and T cell apoptosis [222, 228-230]. Figure 4 provides an overview over L-Arg dependent mechanisms of T cell suppression.

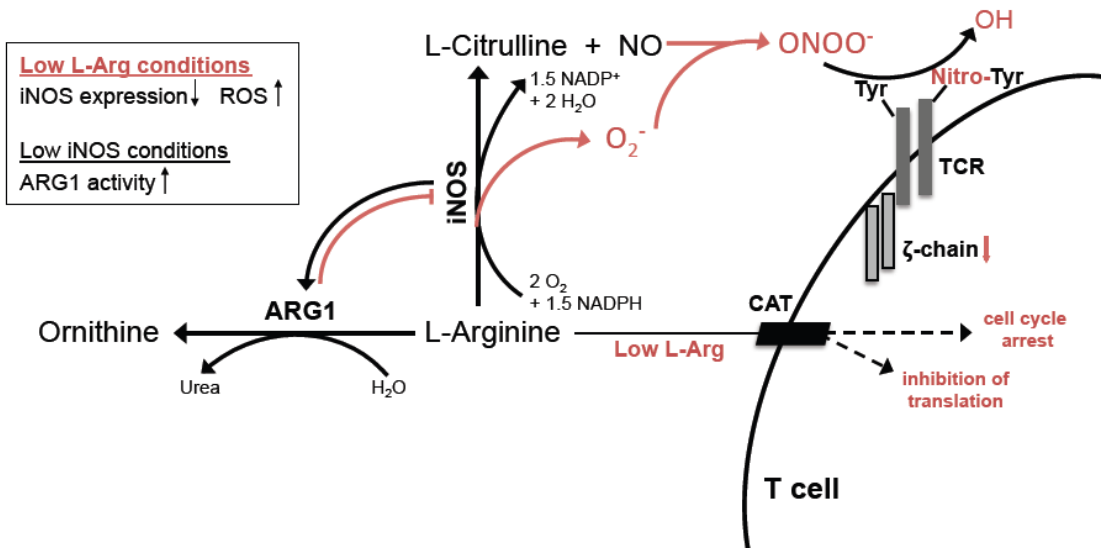


Figure 4: *L-Arg dependent mechanisms of T cell suppression. Effects of low L-Arginine concentrations are highlighted in red.*

2.3.4.2 Co-stimulatory and co-inhibitory receptors and their ligands

Successful T cell activation requires several signals: interaction of the peptide-MHC complex with a TCR of the right specificity (signal 1), co-stimulatory signals (signal 2), and optimally also the presence of stimulatory cytokines (signal 3). Stimulation in absence of co-stimulation will induce tolerance and anergy in the antigen-specific T cell, a mechanism to prevent auto-reactivity. There are a number of co-stimulatory receptors, which mostly interact with ligands belonging to the B7 family of proteins. Members of this protein family can even convey inhibitory signals and consequently dampen T cell activation. So-called co-inhibitory receptors, such as programmed death (PD)-1 and CTLA-4, are often up-regulated after initial T cell stimulation and are thought to function as an 'immunological brake', preventing both activation-induced cell death of the effector cells and collateral damage due to the evoked immune response.

One of the most important co-stimulatory receptors is CD28, which receives signals from CD80 (B7-1) and CD86 (B7-2) and induces effector functions, lowers the T cell activation threshold, and promotes survival [231]. After stimulation, CD28 is down-regulated. This loss of CD28 is sometimes interpreted as a sign of exhaustion since it indicates previous stimulation and the cells' reduced ability to receive co-stimulation [232]. However, CD28 expression is also decreased as a consequence of T cell differentiation, and memory T cells require less co-stimulatory signals [233-235].

As a consequence of T cell stimulation and CD28 engagement, the co-inhibitory molecule CTLA-4 rapidly becomes up-regulated on the surface of activated T cells. CTLA-4 binds CD80 and CD86 like CD28, but with higher affinity, and conveys negative signals that lead to reduced proliferation and cytokine production.

T cell stimulation also induces the expression of PD-1, a co-inhibitory receptor that interacts with the ligands PD-L1 (B7-H1), which can be ubiquitously expressed, and PD-L2 (B7-DC), which is expressed on monocytes and DC [231].

The fact that deficiency in CTLA-4 as well as PD-1 is associated with severe autoimmune disease illustrates the importance of this negative regulation of T cell function [231, 236]. It should be noted that CTLA-4 is also expressed by T_{regs} and has been implicated in their suppressive activity (see section 2.3.2).

At this point, the reader will not be surprised to learn that tumors can make use of these inhibitory pathways to control anti-tumor immunity. Many types of tumors have been reported to express PD-1L [237-239] and are therefore able to inhibit T cells that find their cognate antigen on the tumor [240]. Consequently, high expression of PD-1 on tumor-infiltrating T cells or PD-1L on tumor cells has been found to correlate with poor survival in cancer patients [241-244].

As discussed in chapter 2.3.2, an anti-CTLA-4 antibody was recently approved for the treatment of metastatic melanoma [245]. 'Taking the brake off the immune system' has proven to be a successful strategy to induce immune-mediated control of cancer, and future clinical trials combining anti-CTLA-4 antibodies with immunotherapy are eagerly awaited. Not entirely surprising,

treatment with anti-CTLA-4, especially in subsequent therapy responders, is associated with autoimmune side effects. Though they can be severe, these immune-related adverse events can be managed with steroids without apparent impairment of anti-tumor immunity [246]. Similar trials are underway to elucidate the *in vivo* activity of PD-1 blockade in humans, which has shown some promise in pre-clinical cancer models [247-249].

2.3.4.3 Immunosenescence

Telomeres are the end sections of chromosomes that consist of repetitive DNA sequences. As a consequence of DNA replication, these structures become shortened with each cell division. Telomere length can therefore be used as a measurement of 'cell age' or past replicative activity. If telomeres become too short, the risk of DNA damage increases and the cell will usually undergo cell cycle arrest to protect itself from mutations. Some highly dividing cells express the enzyme telomerase that can extend the telomeres and prevent them from becoming too short. Even tumor cells often express telomerase, which helps them to maintain their limitless replication potential.

With increasing age, the immune system becomes exhausted. This is in part due to the fact that thymic output steadily decreases with age and causes the number of 'fresh' naïve T cells introduced into the circulation to decline [250]. At the same time, exposure to different antigens during our life time can exhaust the pool of naïve T cells, and their slow but continuous turnover reduces T cell **telomere** length over time [250]. Even signaling through T cell surface receptors becomes impaired. These events are summarized under the term immunosenescence [251]. Aging is also associated with a continuous low grade inflammation, cleverly termed 'inflamm-aging', that features many of the key mediators of tumor-mediated immune subversion, such as IL-6, IL-1, T_{regs}, and ROS [251].

Newer data suggest that aging alone is not sufficient to induce immunosenescence, but that it instead could be a consequence of chronic viral infections that becomes more likely with age. For example, about 50% of the population become infected with CMV during childhood, and seroprevalence reaches >85% at the age of 85 [252, 253]. Population-based studies performed in Sweden since the 1980s indicate that 100% of elderly individuals exhibiting an 'immune risk profile', including a characteristic shift from naïve to memory T cells, were infected with CMV, but only 85% of the non-risk group [253]. Similar immunosenescence patterns have even been observed in individuals suffering from HIV infection [254] or cancer [255], suggesting that not old age, but chronic antigen exposure is the likely cause of T cell senescence.

Immunity in very old individuals is currently not well studied, despite the fact that they represent an increasing population in our society. This might represent an important failure of public health evaluation, as studies from the past decade showed that for example individuals with an immune risk profile were less able to respond to vaccination and faced increased rates of mortality [256, 257].

In cancer patients, the concept of senescence induced by chronic antigen stimulation is somewhat puzzling, since the amount of immunogenic antigen supplied by a tumor appears to be low. Furthermore, the frequencies of

antigen-specific T cells, even in immunized, clinically responding patients, are negligible compared with those that can be induced by viral infections [7, 258, 259]. It is possible that the increase in memory T cells instead reflects bystander expansion of this population, for example due to cytokine production by tumor-associated cells.

3 RESULTS AND DISCUSSION

The four articles included in this thesis all deal with tumor-induced immune alterations, but can roughly be divided into two topics: i) tumor-induced immune suppression by MDSC in melanoma patients (Paper I and II); and ii) tumor-dependent changes in T cell phenotype and function in patients with early-stage breast cancer (Paper III and IV). The information provided in the following sections should help the reader understand what was studied in the papers and why, as well as the more general implications of our findings. For details on experimental set-up, results and specific discussion of the findings please see the appended articles.

3.1 MDSC IN MELANOMA

Paper I - *Immature immunosuppressive CD14$^+$HLA-DR$^{-/low}$ cells in melanoma patients are Stat3^{hi} and overexpress CD80, CD83 and DC-Sign*

To study MDSC in melanoma patients, we collected blood samples from 34 patients treated for metastatic (stage III, n=11, or stage IV, n=23) malignant melanoma at Radiumhemmet, Karolinska University Hospital. Initially, we performed flow cytometry on fresh blood samples to identify myeloid populations in melanoma patient blood that were altered in comparison with the blood of healthy, age-matched controls. We found that melanoma patients had a significantly increased frequency of a putative CD14$^+$HLA-DR$^{-/low}$ MDSC population in total PBMC. Accumulation of CD14$^+$HLA-DR$^{-/low}$ cells was only observed in patients with active disease, but not in patients that were tumor-free at the time of blood sampling. The increase in CD14$^+$HLA-DR$^{-/low}$ cells was due to the decreased expression of HLA-DR on CD14$^+$ cells, as the total frequency of CD14$^+$ cells (as well as that of CD3$^+$ T cells and of putative Lin$^-$ CD33$^+$HLA-DR$^-$ MDSC) was similar in patients and controls.
We therefore decided to investigate the suppressive activity of the CD14$^+$HLA-DR$^{-/low}$ population. Cells of this phenotype were isolated from fresh blood samples of melanoma patients or healthy controls and titrated into autologous T cell cultures stimulated with anti-CD3/CD28 beads. We found that CD14$^+$HLA-DR$^{-/low}$ cells from melanoma patients strongly suppressed proliferation and IFN-γ production of CD4$^+$ and CD8$^+$ T cells in this setting and therefore deserved to be termed MDSC. Suppressive activity was significant at MDSC:T cell ratios similar to those observed in the blood of cancer patients. Although no side-by-side comparison was performed, the T cell suppressive activity of MDSC appears to be similar or slightly higher than that of T_{regs} in similar experimental set-ups [51]. CD14$^+$HLA-DR$^{-/low}$ cells from healthy controls suppressed autologous T cells only when added in very high numbers, but not at ratios observed in blood of melanoma patients and, more importantly, not at the much lower CD14$^+$HLA-DR$^{-/low}$:T cell ratios observed in the blood of these healthy controls. These results indicate that CD14$^+$HLA-DR$^{-/low}$ MDSC accumulate in melanoma patients and exhibit more potent suppressive activity

on a per cell basis than cells of similar phenotype observed in healthy individuals.

Since MDSC can be suppressive through a range of different mechanisms, we measured the expression of candidate suppressive molecules in $CD14^{+}HLA\text{-}DR^{-/low}$ cells from melanoma patients and healthy controls by quantitative polymerase chain reaction (PCR). This analysis revealed that melanoma MDSC overexpressed ARG1 in comparison to $CD14^{+}HLA\text{-}DR^{-/low}$ cells from healthy controls. In agreement with the reciprocal regulation of ARG1 and iNOS, patient MDSC exhibited decreased iNOS expression. We also noted that COX-2 expression was decreased compared to controls. To test whether ARG1 overexpression was responsible for T cell suppression, we added the ARG1 inhibitor nor-NOHA to patient PBMC stimulated with anti-CD3/CD28 beads. This could partially rescue T cell proliferation, but did not increase IFN-γ production, suggesting that the mechanisms affecting these two functional measures are not the same. Interestingly, we noted in a number of patients that addition of the ROS-scavenging molecule catalase also improved T cell proliferation and that patients responsive to ROS scavenging usually also responded well to ARG1 inhibition. This led us to further investigate the oxidative potential of the melanoma MDSC. We found that patient $CD14^{+}HLA\text{-}DR^{-/low}$ cells had increased levels of intracellular oxidation, suggesting that ROS production could contribute to T cell suppression. We did not observe differences in mRNA levels of NADPH-oxidase subunits between patients and controls, though translational or post-translational modification of this enzyme in melanoma MDSC cannot be ruled out. An alternative ROS source lies in L-Arg metabolisms by iNOS while ARG1 is simultaneously overexpressed (see chapter 2.3.3.5.1 and Figure 4). Interestingly, we also found that melanoma MDSC overexpressed STAT3 protein, but not mRNA, compared to controls. $HLA\text{-}DR^{-}$ cells also displayed increased levels of STAT3 phosphorylation, indicating STAT3-signaling activity. A correlation between pSTAT3 and levels of intracellular oxidation was revealed. Since oxidative stress did not seem to induce STAT3 phosphorylation, we believe that ROS production in melanoma MDSC could be controlled by STAT3 signaling. Our report was the first to confirm overexpression of STAT3, an important controller of MDSC activation, phenotype, and function, in human MDSC. Importantly, we could further show that inhibition of STAT3 in MDSC completely abrogated their ability to suppress T cell proliferation and IFN-γ production, suggesting that all suppressive mechanisms exerted by this population are under the control of STAT3.

With regard to ARG1 as one of the main suppressive mechanisms in this study, the finding that T cell suppression was cell-cell contact dependent is somewhat puzzling. In contrast to the intracellular ARG1 in mouse MDSC, human ARG1 can be secreted and should therefore not act in a contact-dependent way. However, it is possible that cell contact is not needed for T cell suppression, but rather for MDSC activation.

While looking for markers that could be useful for the identification of human MDSC, we made the interesting observation that melanoma MDSC exhibited a mixed mature/immature phenotype. Compared to healthy controls, melanoma-derived $CD14^{+}HLA\text{-}DR^{-/low}$ overexpressed CD80, CD83, and DC-Sign. These molecules are normally expressed on mature myeloid cells, such as dendritic

cells, and all seem to be involved in myeloid cell-T cell contact. Though we could not decrease the suppressive activity of MDSC by adding blocking antibodies to CD80, CD83, and DC-Sign, it is possible that complete and/or simultaneous inhibition of these three markers, for example by RNA silencing, would prevent MDSC activation. Furthermore, it is of course possible that additional surface molecules that could be co-regulated with the differentially expressed markers identified by us contribute to the contact-dependent suppressive activity of melanoma MDSC.

In conclusion, we found a significant accumulation of strongly suppressive $CD14^+HLA\text{-}DR^{-/low}$ MDSC in patients with metastatic melanoma. ARG1 was one of the main, but not the only, suppressive mechanism employed. MDSC suppressive activity was dependent on direct contact with the T cells and on STAT3 activity. The phenotype of melanoma MDSC was puzzling, as it did not resemble that of normal or activated monocytes, but contained both elements of immature ($HLA\text{-}DR^{-/low}$) and mature ($CD80^+$, $CD83^+$, $DC\text{-}Sign^+$) myeloid cells.

Paper II - *Myeloid-derived suppressor cells impair the quality of dendritic cell vaccines*

In 2009, we initiated a phase I clinical trial that involves treating melanoma patients with a dendritic cell vaccine and adoptive transfer of autologous, *in vitro* expanded T cells.

Based on the findings of Paper I, we were aware that MDSC can accumulate to high numbers in melanoma patient blood and can pose a significant hurdle to T cell function in this patient population. Since many groups have shown that MDSC are impaired in their ability to differentiate into DC, we worried that high frequencies of $CD14^+HLA\text{-}DR^{-/low}$ MDSC could affect the quality of our DC vaccine, which is generated from predominantly $CD14^+$ cells.

This could be a concern for DC vaccine production in any type of cancer where $CD14^+HLA\text{-}DR^{-/low}$ MDSC have been identified (see Table 2).

To test the hypothesis that MDSC negatively affect DC vaccine production, we first investigated whether $CD14^+HLA\text{-}DR^{-/low}$ cells co-purified with the monocytes used as a starting population for our DC vaccine. As large numbers of cells are needed, melanoma patients enrolled in our clinical trial undergo leukapheresis, followed by elutriation (counter-flow centrifugation) to isolate the monocytes. The ratio of $CD14^+HLA\text{-}DR^{-/low}$ cells to total monocytes was constant in the leukapheresis material and the fraction of purified monocytes, suggesting that almost all MDSC co-purify with the monocytes. This is also in accordance with their related phenotype and identical scatter characteristics in flow cytometry. In order to mimic $CD14^+HLA\text{-}DR^{-/low}$ frequencies representative of healthy individuals and cancer patients with intermediate to high MDSC levels, we separated the monocytes into HLA-DR positive and negative populations and re-mixed them into co-cultures containing 0%, 10%, 20%, 50%, 70%, or 100% $HLA\text{-}DR^-$ cells, see Figure 5.

Frequencies of 0-20% $CD14^+HLA\text{-}DR^{-/low}$ cells, as found in the blood of healthy individuals, can be considered ‘physiological’ levels, while 50% or more represent the ‘pathological’ situation observed in many cancer patients [107].

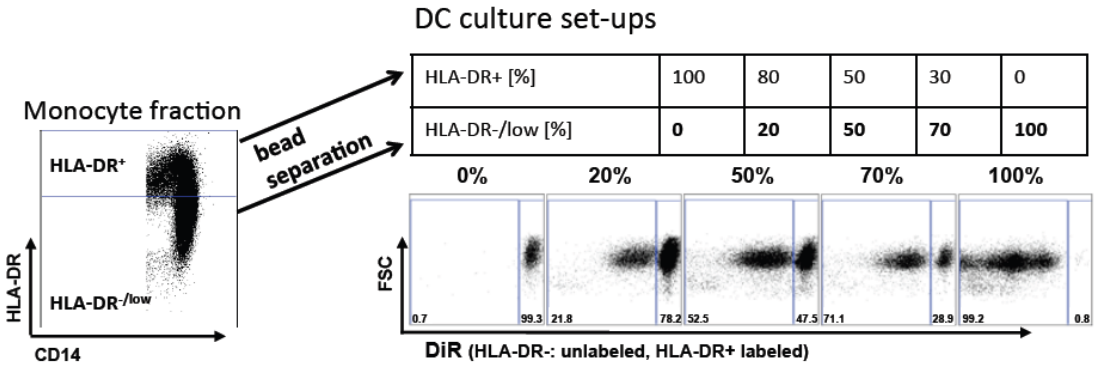


Figure 5. *Set-up up of co-cultures from HLA-DR$^+$ and HLA-DR$^-$ cells isolated from the monocyte fraction. An example of fluorescently labeled HLA-DR$^+$ cells is shown.*

It should be noted that we have previously shown that CD14$^+$HLA-DR$^{-/low}$ cells from melanoma patients and healthy controls differ in their suppressive activity, so that our 'physiological' set-ups could contain more suppressive potential than could be expected if healthy donor monocytes were used.
During the preparation of Paper I, we observed that CD14$^+$HLA-DR$^{-/low}$ rapidly up-regulate HLA-DR when placed in culture. We therefore fluorescently labeled one of the populations to be able to distinguish originally HLA-DR$^-$ and HLA-DR$^+$ cells in the co-cultures (see Figure 5), alternating the labeled population between experiments to prevent bias. Co-cultures were differentiated to DC according to the standard operation procedures (SOP) of our own phase I clinical trial and the phase II clinical trial of our collaborator Dr. Salazar-Onfray [260].
For the outcome of these cultures, we hypothesized four different scenarios:
i) no effect of MDSC on DC generation, ii) preferential death (or survival) of the CD14$^+$HLA-DR$^{-/low}$ population, resulting in reduced DC yield, iii) reduced maturation and functionality of DC generated in presence of MDSC, or iv) a combination of ii) and iii).
The viability of the generated DC was generally high, though the yield strongly varied between experiments. No effect of MDSC frequency on viability and yield could be detected. Moreover, the frequency of HLA-DR$^-$ cells observed in all cultures was very close to what was expected based on the culture set-up.
Analysis of the phenotype and function of mature and immature DC revealed that there was a dose-dependent, negative effect of CD14$^+$HLA-DR$^{-/low}$ on the resulting DC. DC generated in presence of high CD14$^+$HLA-DR$^{-/low}$ frequencies were less mature, had reduced capacity to take up antigen, migrated less efficiently, and induced lower levels of IFN-γ when used to stimulate allogeneic T cells. These effects were most pronounced when 50% or more CD14$^+$HLA-DR$^{-/low}$ were added to the culture. Exposure of the DC to maturation signals reduced the negative effect exerted by the presence of CD14$^+$HLA-DR$^{-/low}$ cells, suggesting that strong stimulation can partially restore normal DC differentiation. Interestingly, the reduced maturation and functionality of DC generated in the presence of high frequencies of HLA-DR$^-$ cells was not only due to the impaired differentiation potential of the CD14$^+$HLA-DR$^{-/low}$ cells.

Comparison of originally HLA-DR$^+$ cells cultured with physiological or pathological levels of CD14$^+$HLA-DR$^{-/low}$ cells revealed that even this population was negatively affected when >50% HLA-DR$^-$ cells were present in the co-culture. The observed differences could not be explained by oxidative stress and cytokine content in the cultures, though increased levels of IL-6 and MIP-1β were measured in supernatants of cultures containing pathological concentrations of CD14$^+$HLA-DR$^{-/low}$ cells.

Due to the high cell requirements of these experiments, only patients enrolled in our clinical trial were investigated and their number remains low due to a slow recruitment rate that is caused by the extensive cell culture that is required for each patient. Interpatient variability was quite high, a fact that became particularely obvious since most experiments were only performed with 3-4 patients. On the other hand, the same trends of MDSC-dependent impairment of DC vaccine quality could be observed across different patients and experiments, and in DC generated according to two different protocols. These SOPs differ in the medium and culture vessel used, as well as in the culture period and the maturation stimulus, but generate comparable results. Based on these findings, the negative effect on DC differentiation and maturation exerted by CD14$^+$HLA-DR$^{-/low}$ cells seems rather robust.

In conclusion, it was possible to generate DC vaccine products that fulfill the general release criteria of our clinical trial protocol, even if high MDSC frequencies were present in the starting material. However, both maturation and essential functions of the resulting DC were suboptimal, suggesting that such vaccines could be less efficient inducers of anti-tumor immunity *in vivo*, particularly if immature DC are used for vaccination. We suggest to monitor the frequency of circulating MDSC in enrolled patients and test depletion of CD14$^+$HLA-DR$^{-/low}$ cells if they represent >50% of the total CD14+ population. This could potentially improve vaccination efficacy in cancers such as melanoma, hepatocellular carcinoma, lymphoma, and prostate cancer, where CD14$^+$HLA-DR$^{-/low}$ have been reported.

Unfortunately, depletion of CD14$^+$HLA-DR$^{-/low}$ cells might prove technically difficult. It will require fluorescence-assisted cell sorting, which is currently challenging under good manufacturing practice (GMP) conditions, or positive selection of CD14$^+$HLA-DR$^+$ cells, which could lead to unwanted cell activation. Alternatively, *in vivo* depletion or differentiation of CD14$^+$HLA-DR$^{-/low}$ cells could become an option once more MDSC-targeting drugs reach the clinic.

3.2 T CELL DYSFUNCTION IN BREAST CANCER

The findings presented in Paper III and IV are the first results from a large study where we assessed the immunological status in the blood, lymph nodes, and tumors of patients with early-stage breast cancer.

Studies on tumor-immune interactions have been performed predominantly in the blood of patients with advanced cancers. Though practical and informative, this approach has several flaws:

i) One of the important aims in studies of tumor-mediated effects on the immune system is to understand why tumor immunotherapy frequently fails

and how it could be improved. Current immunotherapy trials are often performed in patients with advanced cancer that have exploited all other therapeutic options. However, patients with early-stage cancer or those with no measurable tumor or minimal residual disease after first-line therapy are the ones most likely to benefit from immunotherapy. The rationale behind this is that small and early-stage tumors will have experienced less immunoediting and will exert less immunosuppression. Furthermore, this patient population will generally be younger, in better health, and has experienced fewer complications due to treatment. Consequently, one can expect better T cell responses as there is no strong tumor-mediated immunosuppression, and T cells have a better chance to eradicate smaller tumors.
ii) While systemic consequences of tumor-induced immune alterations will be visible in the circulation, peripheral blood can only provide a snapshot of what might be going on in the immune system. T cell priming usually occurs in the lymph nodes and tumor-immune interactions will first take place in the primary tumor and later at sites of metastasis. This suggests that tumor-mediated immunosuppression will be strongest in close proximity to the tumor and decrease with distance. This is in line with previous reportes by our group on immunosuppression in patients with advanced colorectal cancer [210].
We thought that early-stage breast cancer patients represented an interesting population for the study of tumor-mediated immune alterations, because they are usually treated with, and often cured by, surgery, allowing us to compare the immune status in the blood before and after surgery. During surgery, the first tumor-draining lymph node, the 'sentinel' lymph node (SLN), is removed for diagnostic purposes. If a metastasis is detected in the SLN, additional LN are removed from the axilla. This set-up allowed us to collect material from the tumor, as well as from tumor-draining lymph nodes with or without metastasis and, in very few cases, additional non-sentinel lymph nodes.
We set out to test the following hypotheses:

- patients with early-stage breast cancer show signs of immuno-suppression when compared with healthy controls
- surgical tumor removal alleviates tumor-mediated immunosuppression
- immune cells in close proximity to the tumor exhibit more signs of immunosuppression or immunosuppressive potential than those located more distantly; e.g. T cell suppression in tumor > SLN > non-SLN ≥ peripheral (preoperative) blood

Blood samples were collected before and after surgery from 43 female breast cancer patients (stage 1, n=20; stage 2, n=23; stage 3, n=1). Pre- and postoperative blood samples from ten women operated for benign tumors in the breast, such as papillomas or fibroadenomas, served as controls. On the day of surgery, samples from the tumor, the SLN and, if available, additional lymph nodes, were collected from the breast cancer patients. All samples were analyzed by multi-parametric flow cytometry freshly or after short-term culture. Remaining PBMC as well as serum and plasma samples were stored for future use.

Paper III - *Tumor-dependent down-regulation of the ζ-chain in T cells is detectable in early-stage breast cancer patients and correlates with immune cell function*

Statistical analysis revealed that ζ-chain expression on circulating $CD4^+$ and $CD8^+$ T cells was significantly down-regulated in breast cancer patients compared with healthy controls in preoperative blood. This was most pronounced in patients with stage 2 disease. NK cells from stage 2 breast cancer patients showed a trend towards reduced ζ-chain expression compared with controls, though this was not significant. While ζ-chain levels remained constant in healthy controls, they increased significantly in $CD4^+$ and $CD8^+$ T cells as well as NK cells in post- compared with preoperative blood samples of breast cancer patients. Again, the strongest differences were observed in stage 2 patients. Postoperative ζ-chain levels of cancer patients resembled those observed at all time points in the healthy controls, indicating that ζ-chain levels were normalized by tumor removal. Interestingly, ζ-chain levels in $CD4^+$ and $CD8^+$ T cells as well as NK cells were lowest in the tumor, somewhat higher in the SLN (in T, but not NK cells), and highest in peripheral blood in all studied populations. This is in support of our hypothesis that immunosuppression is strongest within the tumor and decreases with distance from the lesion. Tumor-associated T cells in stage 2 tumors showed lower levels of ζ-chain expression than in stage 1 tumors, supporting the theory that tumor progression is paralleled by immune escape/immune subversion.

As it could be detected in the blood as well as in tissues, immunosuppression, manifested as ζ-chain down-regulation, was systemic. This was further illustrated by the strong correlation between ζ-chain levels found on $CD4^+$ and $CD8^+$ T cells and NK cells.

Loss of ζ-chain expression is usually associated with decreased lymphocyte function. We found that low ζ-chain expression in $CD4^+$ and $CD8^+$ T cells as well as in NK cells correlated with low production of IL-2 and TNF-α in $CD8^+$ T cells. This is similar to what has been reported in other cancers [206, 261], though it is possible that we underestimated T cell dysfunction due to strong *in vitro* stimulation. When these experiments were designed we reasoned that a short, unspecific stimulation of peripheral blood-derived T cells would induce production of cytokines reflecting the *in vivo* status of the T cell, e.g., tumor-mediated Th2 skewing. However, it is possible that the stimulus given was too strong to detect subtle differences in T cell activation. Furthermore, in this particular study, a TCR-dependent stimulus could have given better insight into T cell dysfunction caused by reduced ζ-chain signaling. Unfortunately, TCR-mediated antigen-specific stimulation is difficult to achieve in this group of patients due to their unknown HLA profile and the lack of a universally expressed breast cancer antigen. Of note, proliferation assays performed on the same samples did not reveal any tumor- or ζ-chain-dependent differences, despite the use of TCR-dependent stimulation with anti-CD3/CD28 beads in these experiments.

We also observed that patients with low T cell ζ-chain expression had an increased frequency of potentially exhausted $CD28^-$ effector memory T cells in the blood. In general, breast cancer patients had significantly fewer $CD28^+$ T_{em}

cells, suggesting that circulating T cells of breast cancer patients had experienced more stimulation and could be less responsive to co-stimulation. This is interesting as regards the results published by Pages *et al*. showing that the presence of tumor-infiltrating $CD28^+$ T_{em} predicted increased survival in colon cancer patients [262].

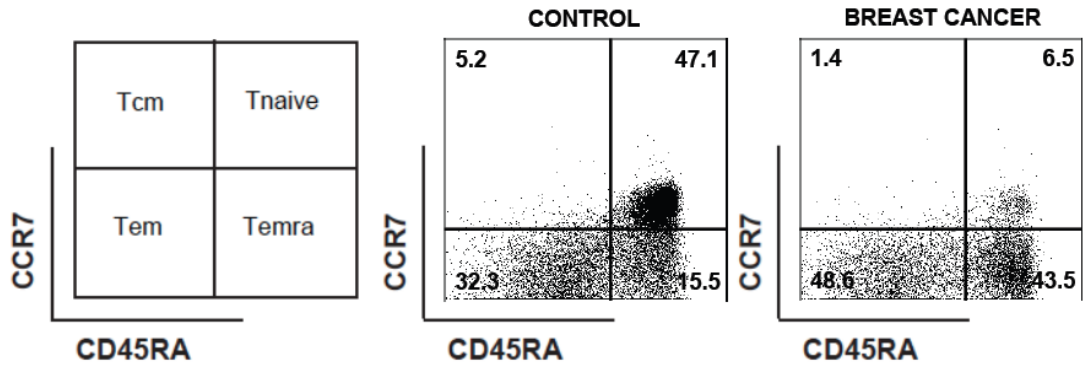
Paper IV - *Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early-stage breast cancer patients*

Motivated by our finding of T cell dysfunction in form of ζ-chain down-regulation, we looked in more detail into changes observed in T cell subpopulations of breast cancer patients.

A comparison of total $CD3^+$ or $CD4^+$ and $CD8^+$ T cells did not reveal any differences between breast cancer patients and controls. However, breast cancer patients with lymph node metastasis had higher frequencies of $CD8^+$ cells in total $CD3^+$ T cells in their blood and in the tumor than those without metastasis. Within the tumor, higher tumor aggressiveness (=higher Elston grade) also correlated with increased $CD8^+$ T cell frequencies. $CD4^+$ T cells usually showed a corresponding decrease, though this did not become significant. This is probably due to the fact that, besides single-positive $CD4^+$ and $CD8^+$ T cells, there are also low frequencies of double-positive and double-negative populations. Even though none of these showed differences similar to those observed in the $CD8^+$ population, small shifts in these subsets could prevent differences in $CD4^+$ T cells from becoming significant.

We further found that peripheral blood and tumor-associated $CD8^+$ T cells in cancer patients contained more T_{em} and T_{emra} cells. Figure 6 gives an example of the distribution of naïve and memory $CD8^+$ T cells in the blood of a breast cancer patient and a healthy control and summarizes some of the key features of T cells at different stages of differentiation. Briefly, using CD45RA and the lymph node homing receptor CCR7, naïve (T_{naive}, $CD45RA^+CCR7^+$), central memory (T_{cm}, $CD45RA^-CCR7^+$), effector memory (T_{em}, $CD45RA^-CCR7^-$), and terminally differentiated memory cells (T_{emra}, $CD45RA^+CCR7^-$) can be distinguished [263, 264]. It is still debated whether T cell differentiation is linear and how it is controlled [265], but it is generally accepted that T_{naive} and T_{cm} can enter the lymphatic system where they can get primed or rapidly expand in response to antigen re-encounter, respectively. T_{em} and T_{emra} cells on the other hand recirculate through the blood and can only enter lymphoid organs through afferent lymphatic vessels, e.g., when drained from a site of infection. T_{em} and T_{emra} cells enter sites of peripheral inflammation after receiving chemokine signals and can rapidly exert cytotoxic activity upon antigen encounter.

The predominance of memory cells in the tumor is therefore not entirely surprising. Interestingly, T_{em} became the dominant population in tumor-associated T cells with increasing Elston grade, while $T_{naïve}$ and T_{emra} cells decreased, suggesting that T cell subset composition or tumor infiltration is controlled by tumor-derived factors.



	$T_{naïve}$	T_{cm}	T_{em}	T_{emra}
CCR7	+	+	-	-
CD45RA	+	-	-	+
CD28	+	+/-	-	-
CD27	+	+	+/-	-
CD62L	+	+	+/-	-
Cytotoxicity	-	+/-	++	+++
Homing to	lymphoid tissues	lymphoid tissues	periphery	periphery

Figure 6: *Characteristics of naïve and memory T cell subsets. Dotplots show a representative example of the T cell subset distribution in a breast cancer patient and a healthy control.*

On the other hand, the shift from naïve to memory T cells in the blood of breast cancer patients is striking, as it indicates that $CD8^+$ T cells in breast cancer patients have either encountered more antigen, leading to priming of naïve T cells, or were expanded in a tumor-dependent fashion. It should be noted that T cell subset composition before and after tumor resection were similar, indicating that the expanded memory T cell pool did not contract after tumor removal. While blood-derived T cells in both healthy donors and breast cancer patients exhibited few signs of recent activation, the expression of CD69 in $CD8^+$ T cells and PD1 in both T cells subsets was higher in patient-derived T cells, indicating previous activation. Furthermore, circulating $CD8^+$ T cells in patients exhibited altered homing capacity in comparison with cells of healthy controls. Patient $CCR7^+$ subsets displayed increased expression of CCR5 and CXCR3, suggesting an increased ability to enter non-lymphoid tissues and possibly providing an alternative explanation for the disappearance of naïve T cells from the blood. CCR5 was even more strongly expressed in tumor-associated T cells than those circulating in patient blood, further pointing to this chemokine receptor as a possible molecule allowing T cell recruitment to the tumor. The expression of CCR5 and its ligand RANTES is well studied in breast cancer, where both molecules can be found in the tumor, but not in adjacent healthy tissue [46, 47]. Interestingly, CCR5 has been implicated in breast cancer aggressiveness and progression, suggesting a possible explanation for the altered T cell subset composition in tumors of different grades that merits further investigation. In agreement with Paper III, tumor-

infiltrating T cells exhibited signs of exhaustion in the form of low CD28 expression compared with blood-derived T cells. However, this phenotype is also in agreement with the high number of memory T cells in the tumor, which tend to be CD28 negative and less dependent on co-stimulation. Interestingly, tumor-associated T cells expressed high levels of CD69, suggesting their activation, though other factors such as the low ζ-chain expression reported in Paper III, as well as the increased presence of regulatory T cells in the tumor, questioning their functional capacity.

Overall, changes observed in the blood of breast cancer patients resemble an immunosenescence phenotype with low frequencies of naïve and high levels of memory T cells, especially subsets re-expressing CD45RA and low in CD28. In elderly humans, this phenomenon has been suggested to result from chronic antigenic stimulation, for example due to CMV infection. The majority of patients included in our study would not be considered elderly. Furthermore, the age difference between patients and controls, though it is significant, is unlikely to be associated with significantly higher rates of viral infections in the patients, as infection with chronic viruses such as CMV or Epstein Barr virus (EBV) is nearly maximal in young adults [266]. On the other hand, it seems counter-intuitive to attribute the T cell differentiation observed in cancer patients to tumor-derived antigens. Most tumor-associated antigens described in breast cancer are self antigens that are overexpressed in the tumor. Consequently, immunity to these antigens should be limited by central tolerance. Searching the literature revealed that even in optimal cases, where immune-mediated tumor regression could be achieved, the number of tumor-antigen specific T cells represents a few percent of circulating T cells at best [259, 267, 268]. It is therefore more likely that the increase in memory T cells is not antigen but cytokine dependent, and the observed shift from naïve to memory T cells is an inflation of the memory subset rather than a true decrease of absolute numbers of naïve T cells. Absolute cell counts would be helpful in clarifying this issue, especially since no difference in $CD3^+$ T cell frequency in breast cancer patients was apparent, suggesting that significant expansion of one T cell population should be associated with decrease of another subset. Based on *in vitro* studies of T cell plasticity, it is also unlikely that T_{em} or T_{emra} cells undergo strong expansion in response to cytokines alone. This is a property associated mainly with T_{cm} cells, that can renew themselves, but also give rise to other memory subsets in response to homeostatic cytokine stimulation, e.g., with IL-7 and IL-15 [263]. The mechanisms behind the altered T cell subset composition therefore remain currently unknown. The altered homing capacity observed in some T cell subsets in the blood, and those T cells that had reached the tumor, suggests an alternative mechanisms to shift the subset balance that should be further investigated.

General conclusions for Paper III and IV

Considering that, with one exception, all breast cancer patients investigated in Paper III and IV had stage 1 or stage 2 disease, our observations of tumor-induced changes in the immune system were somewhat unexpected. We find

that tumor-induced immune suppression is not a hallmark of advanced disease, but that local, as well as systemic, T cell dysfunction can even be observed in early-stage breast cancer patients. Early-stage cancer patients are considered most likely to benefit from immunotherapy, due to limited disease burden and low or absent immunosuppression. Our results suggest that immunomonitoring could be useful for selecting patients who are immunocompetent or identifying an optimal time point for the application of immunotherapy, e.g., after surgery, when T cell function has improved. On the other hand, the decreased frequency of naïve T cells in our patient population suggests that successful active immunotherapy might be more difficult than in healthy individuals, as there are less naïve T cells available for priming. On the other hand, identification of tumor-associated antigens recognized by the expanded memory populations would allow the administration of booster immunization or the expansion of antigen-specific T cells *in vitro*.

A technical note on Papers III and IV

Data analysis of the material described in Papers III and IV is still ongoing. We performed a very broad multi-parametric flow cytometry analysis of all available material, covering T cell subpopulations and functions as described above. We also studied regulatory T cell subsets, myeloid populations including potential MDSC and different DC subsets, and additional functional measures such as the expression of ARG1, iNOS, and the production of oxidative stress. All these measurements have been correlated with clinical parameters and are now studied for their interconnection. Expression of some markers will be followed up by immunohistochemistry. Unfortunately, immunohistochemistry allows co-staining of only two markers at a time and therefore makes the identification of specific subpopulations difficult. However, the spatial distribution of stained cells in immunohistochemistry will add information to the data collected by flow cytometry.

While it was informative, this project was characterized by time-consuming sample handling and very tedious and work-intensive data mining. Technical advances, e.g., in modern cytometry, have lead to an exponential increase in the amount of data that can be generated. This is a great advantage, as more information can be extracted from lower amounts of material and the simultaneous analysis of many immune parameters will allow a better understanding of how things are connected. On the other hand, there is a great need for tools that allow us to handle and understand such large amounts of data, and the project described above is currently somewhat limited by the lack of appropriate data mining software.

In an effort to approach thousands of variables in an unbiased way, we have attempted to perform unsupervised clustering analysis on our data set, similar to analysis of microarray data. We immediately noticed that the process of extracting and entering data needs to be streamlined to allow such analysis to be performed in a timely manner. Automatic data analysis software is becoming available, but is still in need of validation. Furthermore, gating strategies identifying populations of interest are still difficult without human supervision and input. After some first attempts to perform clustering analysis

on the data set introduced above, we are also still debating if gated frequencies of subpopulations or mean fluorescence intensity values of total cells and their subpopulations would be most suitable for this kind of analysis. A look on the cover of my thesis will give you an appreciation for the complexity of these analyses. The heat map shown on the cover is part of a clustering analysis performed with all patients and mean fluorescent intensity values of preselected cell populations, thus including only a fraction of the data and no clinical parameters at all. These analyses are still in their infancy and are therefore unfortunately not yet reflected in the articles presented here. We will, however, continue to explore data clustering methods for high-throughput analysis of flow cytometry data, using the data presented in Papers III and IV for validation.

4 CONCLUSION

As the world's population grows older and lifestyle changes alter our health, the incidence of many cancers has increased, while others are disappearing. Fortunately, some cancers have become highly treatable, especially if they are discovered early. Surgery remains our most potent weapon for combatting cancer, and chemotherapy has successfully prolonged the life of many cancer patients. However, some cancers, such as melanoma, remain difficult to treat and are associated with a very poor prognosis. Though it is currently highly individualized and very expensive, immunotherapy can represent an additional therapeutic option for patients with cancer.

Everyone who has read this thesis (small test: the first 5 persons to tell me how many times I have used the word 'MDSC' on page 18 will get a price), and all of us working with tumor immunology, know that the interactions between tumors and the immune system are manifold. At the time we study these, the tumor has often spread and exerts significant suppression on the immune system. However, there is plenty of evidence suggesting that the immune system can indeed control tumors, even though the cases investigated by us usually represent those in whom the tumor has 'won'.

I have decided to play on the side of the immune system. When I graduated from high school I wrote in my yearbook that (among some other things) I was planning to cure cancer. Though I have not succeeded (yet), I have certainly tried to add to our understanding of tumor-immune interactions. I believe that adding small bits to our basic understanding of anti-tumor immunity and tumor-mediated immune subversion is very important, as it provides a knowledge base that can be exploited for designing better cancer therapies. I was lucky to perform my PhD during a time when enthusiasm for immunotherapy was being rekindled and several immunotherapeutic products finally started to reach the clinics. In the year 2010, Sipuleucel-T, the first therapeutic anti-cancer vaccine in humans, was approved for use in advanced stage, hormone-refractory prostate cancer. In March of 2011, the American food and drug administration approved Ipilimumab, a monoclonal antibody targeting CTLA-4, for the use in metastatic melanoma, where it has been shown to nearly double 1-year survival rates in several phase 3 clinical trials.

Even though the complexity of the immune system can sometimes be dazzling, clever tweaking of the system, most likely by a combination of several conventional and immunological therapies, should be able to shift the balance in favor of anti-tumor immunity. I believe that immunotherapy will become more successful as we better understand tumor-immune interactions and which patients are able to benefit from such treatments. The complex, time-consuming and expensive manufacturing, particularly of cellular products, has deterred many companies from developing immunotherapies. However, as culturing procedures are streamlined and more off-the-shelf solutions, e.g., tumor antigen-specific T cell receptors, become available, more patients will be able to benefit from immunotherapy in the future.

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