General introduction:

targeting myeloid suppression in cancer immunotherapy

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Cross-talk between tumor and myeloid cells:
how to tip the balance in favor of antitumor immunity.
General Introduction - Targeting myeloid suppression in cancer immunotherapy

Successes and hurdles in the immunotherapy of cancer
Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths which is around 13% of all deaths in 2008. Although improvements have been made in surgical techniques, radiotherapy and adjuvant chemotherapy, a large number of patients that have initially been cured, eventually relapse and die of this disease. Therefore, there is a need for additional treatment strategies to target cancer cells. One of the most promising methods is cancer immunotherapy. In recent years, exciting new developments have resulted in different immunotherapeutic approaches. Particularly checkpoint inhibitors (against CTLA4 and PD-1) releasing the brakes on anti-tumor T cells have led to clinical success, resulting in durable responses. Unfortunately, only a fraction of the treated patients experience this long-lived clinical benefit. A failure to respond has been linked to the absence of pre-existent anti-tumor immunity and tumor-induced immune suppression, both in the microenvironment and systemically. It therefore remains vital to break this cancer-imposed immune suppression and achieve a level of functional anti-tumor immunity.

Dendritic cells (DC) are the key orchestrators of the adaptive immune system. DC have an outstanding ability to capture, process and present antigens to activate naive T cells. They also have the ability to regulate the nature of the T cell response by providing appropriate costimulatory signals that dictate either immunogenic or tolerogenic T cell stimulation. These unique features make targeted manipulation of DC an attractive approach for inducing and enhancing immune responses against cancer. The studies described in this thesis were designed to investigate ways in which to selectively target and modulate DC in support of tumor immunotherapy.

Disturbed myeloid cell biology in cancer: immune suppression gains the upper hand
DC are part of the myeloid cell lineage which is vitally involved in tissue homeostasis, defense against pathogenic invasion and induction and regulation of inflammation, as well as activation or suppression of the adaptive arm of the immune system. In cancer, a disturbed balance in myeloid subset development and functioning leads to a profound state of immune suppression, both regionally, in and around tumors, and systemically, in more advanced disease stages. In order for any immunotherapy to be effective, this immune suppression will have to be eliminated.

The next paragraphs describe the different myeloid subsets and their specific functions as well as their disturbed development and functionality under tumor conditions. In subsequent paragraphs the tumor-derived factors and molecular mechanisms that are implicated in this disturbed myeloid development will be discussed. Finally, possibilities for therapeutic intervention through DC targeting and the studies that make up this thesis will be outlined.

Dendritic cells
DC provide the crucial link between the innate and the adaptive immune system, and operate
at the most fundamental levels of regulation in numerous immune processes. DC stem from a common CD34+ BM-derived precursor, and can differentiate into various subsets, which can be myeloid (i.e., conventional DC (cDC)) or plasmacytoid (pDC) in nature. Human pDC are characterized by the expression of the IL-3 receptor (CD123) and the C-type lectin BDCA-2/CD303, whereas cDC express high CD11c levels. In addition, under inflammatory conditions, certain DC subsets can quickly develop from CD14+ monocytes. From the blood, DC precursors seed peripheral tissues, where they develop into so-called immature DC. Upon their proper activation through pro-inflammatory and/or infectious stimuli, they reach end-stage maturation (characterized by the expression of the maturation marker CD83 and other costimulatory molecules CD80 and CD86, See Figure 1), accompanied by their ability to migrate to secondary lymphoid organs and activate (naive) T cells in an antigen-specific manner.

Figure 1. Dendritic cell differentiation and maturation. DC precursors are able to differentiate into immature DC in response to different cytokines. After exposure to activation-inducing stimuli these immature DC will become mature and able to prime and activate T cells (adapted from 1).

Conventional DC have an important sentinel function in vivo, and express a range of microbial pattern-recognition receptors in aid of this, such as C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). Excessive tissue damage or signs of microbial invasion can induce maturation and migration of cDC to lymph nodes (LNs) to elicit an adaptive immune response. Efficient T cell responses are only induced when the cDC are matured and have started to produce high levels of proinflammatory cytokines, such as IL-12p70, TNFα and type I interferons. In particular IL-12 is important for Th1 polarization and the activation of cytotoxic T lymphocytes (CTLs). In sharp contrast, immature cDC fail to induce an effective cell-mediated immune response, since they express low levels of MHC and costimulatory molecules. In fact, the interaction between immature cDC and T cells will result in T cell anergy and the induction of Tregs. Moreover, it has been reported that cDC can also induce T cell anergy after priming with the immune-suppressive cytokine IL-10.

Besides cDC, pDC also play a role in the generation of innate and adaptive immune protection against, for example, viral infection. In the blood (and LNs), they display an immature phenotype, characterized by the C-type lectin BDCA-2/CD303. Of note, studies of both murine and human pDC differentiation have shown that pDC can stem from both myeloid and lymphoid progenitors.
pDC release large amounts of IFNα upon stimulation by CD40 ligand or appropriate TLR ligands (TLR-L) and thereby boost NK cell and CTL responses \(^{13,14}\). In addition, pDC can also directly (cross-)present tumor-derived epitopes and, thus prime tumor-specific CTLs \(^{13,15,16}\). However, pDC that are activated by IL-3 and CD40 ligand stimulation preferentially promote Th2 differentiation, and pDC are even able to suppress immune responses directly by anergizing effector T cells \(^{17}\). Of note, pDC in tumor-draining LNs express high levels of indoleamine 2,3-deoxygenase (IDO) and play an active role in CTL suppression through environmental tryptophan depletion \(^{18}\).

The specific effect of the different DC subsets on immune function in large part is determined by environmental conditions. These issues are currently an important focus of investigation, but more research is needed, in particular, to study the clinical relevance of these subsets in vivo.

**Other myeloid subsets & their roles in inflammation, immunity & tolerance**

In general, mature cells derived from the myeloid lineage are short-lived and must be replaced continuously throughout life. Myeloid cells originate from a self-renewing population of multipotent hematopoietic stem cells (HSCs) present in the bone marrow (BM). Under the influence of certain cytokines, HSCs can differentiate into progenitors of two major leukocyte lineages: the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CLP cells can further differentiate into lineage-specific T lymphocytes, natural killer (NK) cells and B lymphocytes. In the myeloid lineage, CMPs give rise to several immature progenitor cells, including the granulocyte-monocyte progenitor, eosinophil progenitor and erythroid

![Figure 2. Normal myeloid differentiation pathways. The heterogeneous IMC population is indicated by the dashed box. Under certain conditions, IMC can acquire immunosuppressive traits and convert to myeloid-derived suppressor cells. CMP: Common myeloid progenitor; DC: Dendritic cell; HSC: Hematopoietic stem cell; IMC: Immature myeloid cell.](image)
progenitor. Mature descendants of these progenitors circulate in the blood and will migrate to
different peripheral tissues, where they can become activated or, similar to monocytes, further
differentiate (e.g., into macrophages or DC). Figure 2 gives an overview of differentiation
pathways within the myeloid lineage.

In healthy individuals, immature myeloid cells will differentiate into mature macrophages, DC
and granulocytes. By contrast, factors present during trauma, infection or tumor development
are able to inhibit this differentiation, which results in an accumulation of immature myeloid
cells (IMC; see Figure 2) and their development into so-called myeloid-derived suppressor
cells (MDSC), which produce several immune suppressive factors that can effectively interfere
with T cell immunity \(^{19,20}\). For a summary of myeloid subsets with suppressive traits, see Table 1.

### Table 1. Human immunosuppressive myeloid subsets over-represented under tumor conditions.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Phenotype</th>
<th>Main suppressive mediators</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>CD15(^*)</td>
<td>ARG1, IL-10</td>
<td>21-23</td>
</tr>
<tr>
<td>MDSC(^*)</td>
<td>Lin(^{+})HLA-DR(^{-})CD33(^{+})CD11b(^{+})</td>
<td>ROS, iNOS, ARG1, TGF-(\beta)</td>
<td>19,24</td>
</tr>
<tr>
<td>TAM/M2</td>
<td>CD14(^{+})MR(^{+})CD163(^{+})CD103(^{+})</td>
<td>IL-10, decoy IL-1 Receptor-2, ROS</td>
<td>25</td>
</tr>
<tr>
<td>Tolerogenic DC(^*)</td>
<td>CD83(^{-})CD86(^{+})CD80(^{+})PDL-1</td>
<td>IL-10, TGF-(\beta), IDO, coinhibitors (e.g.)</td>
<td>5,12,18</td>
</tr>
</tbody>
</table>

\(^{+}\)In mouse: monocytic MDSC, CD11b\(^{+}\)Ly6G\(^{-}\)Ly6C\(^{+}\) and granulocytic MDSC, CD11b\(^{-}\)Ly6G\(^{+}\)Ly6C\(^{+}\).  
\(^{*}\)Can be CD15\(^{-}\) or CD15\(^{+}\); also a monocyctic subset has been described: CD14\(^{+}\)HLA-DR\(^{-}\)DC-SIGN\(^{-}\)CD80\(^{+}\)CD83\(^{-}\).  
*Can be immature or semimature; characterized by a lack of IL 12p70 release.

ARG1: Arginase-1; DC: Dendritic cell; IDO: Indoleamine 2,3-deoxygenase; iNOS: Inducible nitric oxide synthase; MDSC: Myeloid-derived suppressor cell; ROS: Reactive oxygen species; TAM: Tumor-associated macrophage.

### Monocytes & macrophages

Monocytes account for some of the most important cell types in inflammation. Monocytes
represent 10–15 % of the peripheral blood mononuclear cells (PBMCs). They can enter
peripheral tissues, where they can give rise to macrophages and DC. In general,
macrophages play an important role in tissue homeostasis though the clearance of senescent
cells and the remodeling and repair of tissue after inflammation. Once macrophages enter
inflamed tissue, they acquire distinct phenotypes and physiological activities, determined by
microenvironmental cues. Based on in vitro experiments, the following activation states of
macrophages have been described:

- The classically activated M1 macrophage, which is induced upon stimulation with
  lipo-polyssacharide (LPS) and IFN-\(\gamma\), and is associated with cellular immunity, pro-
  inflammatory cytokine production and microbicidal activity;
- The alternatively activated M2 macrophage, which arises upon stimulation with IL-4
  and/or IL-13, and is linked to tissue repair and humoral immunity. M1 macrophages
  show tumor-rejection properties, whereas M2 macrophages are known to promote
  tumor growth.
• The innately activated subset, induced through Toll-like receptor (TLR) signaling, and characterized by microbicidal activity and proinflammatory cytokine production;
• The deactivated subset, induced through, for example, IL-10 or TGFβ signaling, which displays immune-suppressive features, such as downregulation of MHC class II expression and the production of anti-inflammatory cytokines.

It is not yet clear whether these distinct activation states faithfully represent physiological macrophage subsets, or that macrophages display an even broader range of phenotypes in vivo. Tumors are generally characterized by high levels of macrophage infiltration. These Tumor-associated macrophages (TAMs) most often display an M2- or deactivated macrophage-like functionality, resulting in T cell tolerance and Treg expansion. Moreover TAMs can also directly promote angiogenesis and tumor growth and invasion.

Neutrophilic granulocytes
Polymorphonuclear granulocytes are the most abundant subset of phagocytic cells found in the blood. Neutrophilic granulocytes can rapidly home to, and extravasate at, sites of bacterial infection, and are a highly prevalent leukocyte subset under inflammatory conditions. They phagocytose bacteria (facilitated through Fc- or complement-binding receptors) and degrade and eliminate them through the bactericidal activity of enzymes contained within their characteristic granules. Neutrophilic granulocytes can become over-represented in cancer patients through paraneoplastic myeloproliferation and actually acquire T cell suppressive characteristics, which is associated with a poor prognosis. A report by De Santo et al. described the induction by the acute-phase reactant serum amyloid-A1 (SA-A1) of IL-10 secretion by neutrophils. Of note, SA-A1 levels were increased in patients in advanced stages of melanoma, and SA-A1-conditioned neutrophils acquired a potent T cell-suppressive capacity. Encouragingly, the authors also described the ability of activated NKT cells to inhibit the IL-10 release by neutrophils.

Myeloid-derived suppressor cells
Under chronic inflammatory conditions, impaired macrophage and DC differentiation may lead to the accumulation of MDSC, which also contribute to the regulation of immune responses. In healthy individuals, MDSC comprise only approximately 0.5 % of PBMCs. In cancer patients, they are found at abnormally high rates, probably resulting from paraneoplastic myeloproliferation, as well as disturbed myeloid differentiation. MDSC develop from a heterogeneous population of IMC that have been prevented from differentiating into mature myeloid cells and, for example, under the influence of tumor-imposed conditions, acquire potent immunosuppressive functions.

It has been speculated that MDSC evolved as a regulatory component of the immune system, preventing pathological immune-mediated damage. While MDSC are defined by their main functional aspect (i.e. T cell suppression), their phenotypic identification has been complicated by a lack of specific markers. Our current knowledge of MDSC is mostly based on mouse studies, with human studies lagging behind. In the next two paragraphs, MDSC subsets and their phenotypes in mice and humans will be briefly discussed.
In mice, MDSC are most clearly defined as cells that are positive for the surface molecules Gr1 and CD11b. CD11b is a characteristic marker for macrophages, while Gr1 is a myeloid-differentiation antigen, which consists of two epitopes recognized by anti-Ly6G or anti-Ly6C antibodies. Murine MDSC populations can be divided into two major subsets: CD11b^+Ly6G^-Ly6C^- cells, which have a granulocytic phenotype, and CD11b^+Ly6G^-Ly6C^+ cells, with a monocytic phenotype (Table 1). The cells with a granulocytic phenotype have multilobed nuclei and are thought of as terminally differentiated cells, whereas the monocytic cells have mononuclear nuclei, and are able to further differentiate into macrophages and DC. There are also other markers described for subsets of MDSC, such as the IL-4 receptor-α (IL-4Rα), CD115 (macrophage colony-stimulating factor [CSF] receptor), CD49d, and the costimulatory molecule CD80, but these markers are not present on all MDSC populations. MDSC subsets can also vary in their expression of immunosuppressive mediators. A distinction can be made between subsets that contain arginase, inducible nitric oxide synthase (iNOS) and/or reactive oxygen species (ROS).

Human MDSC are mostly defined as cells that express the common myeloid markers CD33 and CD11b, but lack the expression of markers of more mature myeloid and lymphoid cells and the MHC class II molecule HLA-DR (Table 1). Within this CD11b^-CD33^-CD14^-HLA-DR^- cell population, the expression of CD15 and other markers can vary. A CD14^-HLA-DR^- population of myeloid cells was described in a metastatic melanoma patient, and was shown to express CD83, CD80 and DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) and to exert their suppressive effects through TGFβ- and arginase-mediated mechanisms. A further suppressive mediator found in human MDSC is iNOS. Variations between subsets of MDSC and their suppressive mediators may be explained by their recruitment to different types of tumors, directing the differentiation and functionality of different MDSC types. Of note, there is an urgent need to define tumor-associated myeloid subsets and their specific immune-modulatory characteristics. A case in point was provided by a recent publication from the group of Dr Stephen Rosenberg which called into question the T cell-suppressive qualities of melanoma-infiltrating CD14^-HLA-DR^dim cells, generally accepted in the field as MDSC.

Cancer-related development of myeloid subsets with immune-suppressive traits

Over the past decade, the connection between inflammation and cancer has been well established. The first clues included the notion that tumors often arose at sites of chronic inflammation, and that inflammatory cells could be found in biopsied samples of tumors. Further evidence includes the observations that:

- Inflammatory diseases increase the risk of developing certain types of cancer;
- Signaling pathways involved in inflammation also operate downstream of oncogenic mutations;
- Inflammatory leukocytes and mediators can be found in the microenvironment of all tumors in animal models and humans;
- Targeting of these inflammatory cells or mediators can decrease the incidence and spread of cancer.
As a consequence of this growing body of evidence, the association between inflammation and the risk of cancer development is becoming more appreciated. Perhaps surprisingly, cancer-related inflammation often results in the suppression of effective T cell-mediated anti-tumor immunity, rather than its activation. This is the result of collateral activation of suppressive effector cells, such as Tregs and MDSC. Moreover, inflammatory mediators, released by tumors and their immune infiltrate, influence systemic myeloid differentiation, as well as the functionality and activation of myeloid subsets in the tumor microenvironment. As a result of this aberrant myeloid differentiation and trafficking, decreased numbers of DC are present in the blood of cancer patients compared with healthy donors. Both pDC and cDC can be affected. Reduced numbers of DC were also observed in tumor tissue and tumor-draining LNs. Moreover, these tumor-associated DC were minimally activated and had low expression levels of the costimulatory molecules CD80 and CD86, possibly inducing T cell anergy or tolerance. In keeping with this notion, a low number of mature DC in cancer patients has been associated with a poor prognosis. Concomitant with the reduced number of DC, the population of IMC can be increased in the blood of cancer patients. IMC can convert to MDSC and, as such, have been found to suppress antigen-specific CD8+ T cells in cancer through the production of ROS. Finally, frequencies of neutrophilic granulocytes are often increased in cancer patients, possibly resulting from raised systemic IL-6 levels, inducing a so-called paraneoplastic syndrome. Neutrophilic granulocytes can suppress NK and T cell activation through their expression of arginase-1 and the release of IL-10. Indeed, MDSC-suppressing T cells in patients with renal cell carcinoma (RCC) were identified as a subset of granulocytes.

In summary, cancer-related aberrant myeloid differentiation is characterized by an increase of IMC, M2 macrophages, granulocytes and MDSC, all with T cell-suppressive abilities, whereas rates of T cell-stimulatory DC are decreased. The tumor-induced effects on myeloid differentiation are systemic, indicating that the effects are mediated by soluble (inflammatory) factors. Several years of research have not led to the identification of a single responsible factor, but several possible candidates, an interplay between which most likely causes the observed effects on myeloid differentiation. Of note, the abnormal composition of myeloid subsets in cancer patients may stem both from elevated levels of factors causing proliferation of early myeloid precursors (e.g., IL-6 and granulocyte–macrophage [GM]-CSF) and from factors blocking the differentiation and activation of DC (e.g., IL-10, VEGF and IL-6), with the combination resulting in increased rates of immature suppressive myeloid subsets and reduced frequencies of mature DC. To complicate matters further, in different tumor types, different factors might play a role in this regard.

In the following section, an overview is given of the factors involved in blocked DC differentiation (and collateral accumulation of immature myeloid cells, giving rise to MDSC).
General introduction

Figure 3. Myeloid differentiation under normal and cancer conditions. Red text/arrows and dotted arrows indicate respective over- and under-representation of myeloid subsets and differentiation pathways in cancer. CMPs originate from HSCs and differentiate into IMC, which, under the influence of tumor-derived IL-6, VEGF and associated STAT3 signaling, for example, can be blocked from further differentiation into MO, and further on to MF and DC and, instead, acquire suppressive characteristics, converting to grMDSC and moMDSC. Blocked systemic DC differentiation as well as enhanced granulocyte frequencies in cancer patients, have been linked to VEGF and/or IL-6, respectively. IL-4 and/or IL-13 in the tumor microenvironment can direct MO and MF to an M2 phenotype, while IL-10 can convert tissue-resident and monocyte-derived DC into macrophages with a lack of T cell-stimulatory abilities.

Cancer-associated myeloid suppressive factors

A number of cancer-related suppressive factors have been identified that can interfere with normal myeloid development at different levels (see Figure 3) Five of the major culprits and their detrimental effects are described below.

VEGF

Early in vitro studies, in which neutralizing VEGF-specific antibodies abrogated the negative effect of tumor-conditioned media on the differentiation of DC from HPCs, pointed to the involvement of VEGF in tumor-induced defects in DC differentiation. Subsequent in vivo experiments confirmed the role of VEGF in DC differentiation: recombinant VEGF administration in mice resulted in inhibition of DC differentiation, which was accompanied by an increase in Gr1+ IMC. In addition, the stimulatory effects of FLT3L on DC generation were abrogated when VEGF was co-administered, and tumor-bearing mice showed improved DC differentiation when antibodies neutralizing the effects of VEGF were administered. Correlative data obtained in
humans have supported the role of VEGF in defective DC differentiation in cancer-expression of VEGF has been found to negatively correlate with DC frequencies in tumor tissue and in peripheral blood of cancer patients.

**Gangliosides**
Gangliosides are structurally diverse acidic glycosphingolipids that exist as clusters on the cell surface. They function as signaling intermediates in the regulation of multiple cellular responses, and have several effects on the immune response: they can inhibit antigen processing and presentation, T cell proliferation and the release of Th1 cytokines, thereby skewing immune responses in the tumor microenvironment towards a detrimental type-2 profile. Different tumor types show enhanced production and release of gangliosides. Importantly, gangliosides have also been implicated in tumor-related disturbed myeloid differentiation. The ganglioside GM3 has been described to impair differentiation and function of both CD34+ precursor- and monocyte-derived cDC. Melanoma-derived GM3 and GD3 gangliosides also inhibited phenotypic and functional differentiation of cDC from blood-derived monocytes in a dose-dependent manner. Furthermore, these gangliosides induced DC apoptosis.

**Prostanoids**
Prostanoids, including prostaglandins, are produced from arachidonic acid by the sequential enzymatic actions of cyclo-oxygenases (e.g., constitutively expressed COX-1 and inducible COX-2) and respective synthases. These inflammatory mediators are formed and released in response to various stimuli, and serve to maintain local tissue homeostasis. They can exert both pro- and anti-inflammatory effects, particularly on DC, depending on the nature of maturation signals. Both endogenous and exogenous prostaglandin-E2 (PGE2) plays a regulatory role in the immune response via modulation of IL-12 production by DC. It has also been demonstrated that prostaglandins (mainly PGE2), are involved in tumor-induced immune suppression. Over-expression of the inducible COX-2 enzyme is a common feature in human lung, colon, breast and prostate cancers. Interruption of the PGE2 pathway in vivo has been shown to delay and/or prevent tumor progression in mouse models. For example, mice treated with COX-1 and -2 inhibitors showed a delay in primary tumor onset and tumor progression. In addition, treatment with these inhibitors reduced metastatic disease in mice. These effects were, at least in part, attributable to normalized DC and T cell functions, resulting in an effective anti-tumor response. We found that primary colon carcinoma-conditioned media hampered the differentiation of CD14+ and CD34+ precursors more effectively than conditioned media of established colon carcinoma cell lines, owing to high levels of PGE2 specifically released by primary colon carcinomas. The DC inhibitory effects of these primary tumor-conditioned media were abrogated when they were prepared in the presence of indomethacin, a COX-1/-2 inhibitor. Moreover, prostanoids were identified as the main causative factors of disturbed DC differentiation from monocytes in supernatants from primary human tumors of diverse origins, underlining the universal significance for solid tumors of the DC-inhibitory influence of prostanoids.
**IL-6**

IL-6 is a key tumor growth-promoting and anti-apoptotic inflammatory cytokine, which has a clear pro-tumoral role in multiple myeloma, for example\(^\text{61}\). IL-6 has also been described to mediate tumor-induced inhibition of DC differentiation. RCC cell lines were found to release large M-amounts of IL-6 and M-CSF, which concertedly inhibited the differentiation of CD34\(^+\) precursors into DC and induced the generation of immature monocyctic cells\(^\text{84}\). Antibodies neutralizing the effects of IL-6 and M-CSF could abrogate these inhibitory effects on DC differentiation\(^\text{84}\). Park et al. demonstrated that IL-6 also suppresses DC maturation in vivo, employing IL-6-/mice\(^\text{85}\). Human in vivo data confirmed the role of IL-6 in DC suppression. Defective DC functioning in multiple myeloma patients was shown to be partially due to IL-6\(^\text{86}\). We found evidence of an important role of tumor-derived IL-6 in the suppression of human DC differentiation in three separate studies:

- In combination with PGE2, IL-6 in supernatants from primary colon tumors blocked DC differentiation from CD34\(^+\) precursors\(^\text{83}\);
- In advanced RCC patients treated with sunitinib, we observed a significant inverse correlation between serum IL-6 levels and frequencies of the BDCA1/CD1c\(^+\) cDC subset in peripheral blood\(^\text{54}\);
- Glioblastoma-derived IL-6 was identified as the perpetrator of the inhibition of Langerhans cell (LC) and DC differentiation from CD34\(^+\) precursors in vitro\(^\text{87}\).

These findings collectively point to IL-6 as a major culprit in systemic tumor-induced inhibition of DC differentiation. Therefore, we postulate that systemic IL-6 blockade might be a beneficial addition to any (DC-based) immunotherapeutic approach. Beside neutralizing anti-IL-6 monoclonal antibodies, the tyrosine kinase inhibitor (TKI) sunitinib might also be a useful tool in this regard, since we observed that the inverse correlation between serum IL-6 levels and BDCA1/CD1c\(^+\) cDC rates was abrogated upon 4 weeks of sunitinib treatment, but reasserted itself after a subsequent 2-week-off period\(^\text{54}\). This observation is suggestive of reduced IL-6 responsiveness of the BDCA1/CD1c\(^+\) cDC subset or its precursors due to the downstream TKI activity of sunitinib, although the involved tyrosine kinase targets remain to be identified.

**IL-10**

The inhibitory effect of IL-10 on DC differentiation has been well recognized. IL-10 has been shown to block the differentiation of cDC from monocytes, while promoting their maturation into macrophages\(^\text{88,89}\). Furthermore, functional activities of LC, which are the cDC localized in the epidermis, and of cDC developed from CD14\(^+\) and CD34\(^+\) precursors in vitro, were inhibited by IL-10\(^\text{12,90,91}\). In tumor-bearing mice, tumor-induced IL-10 appeared to be responsible for suppressing the function of activated DC; additional evidence was provided by the improvement of splenic DC function in tumor-bearing IL-10-deficient mice\(^\text{92}\). Beside tumor cells, tumor-associated lymphocytes can produce IL-10, with a subsequent negative effect on DC differentiation\(^\text{93}\). Increased circulating levels of IL-10 have been reported in cancer patients compared with healthy donors\(^\text{94,95}\). However, no correlation was observed between serum IL-10 levels in cancer patients and defects in DC differentiation\(^\text{54,56}\). Of note, IL-10 is
the only known factor with the capacity to block activation/maturation of fully differentiated cDC\textsuperscript{12}, a process occurring in peripheral tissues and preceding their migration to the LNs. \textit{In vitro} studies suggest that upon maturation cDC have become resistant to these inhibitory effects of IL-10\textsuperscript{12}. Taken together, these observations seem to point to a specific role for IL-10 in suppressing the local development and functions of immature cDC within (tumor) tissue microenvironments.

Tumor-derived suppressive factors bind various receptors on myeloid cells but down-stream signals may converge in shared pathways. Mostly from mouse studies, the JAK2/STAT3 signalling pathway has emerged as a “master switch” of tumor-induced immune suppression. Through functional kinase profiling, Kinase Inhibitors (KIs) and RNA interference (RNAi), additional signalling elements have been and will be identified as involved in the regulation of DC development and suppression. Clinically applicable small-molecule KIs and RNAi-based therapeutics allow for targeting not only of tumors, but also of their stroma, and should facilitate re-programming of tumor-modulated myeloid cells, DC in particular, in support of anti-tumor immunity. Some of the signaling cascades known to play a role in DC development and physiology are briefly described below.

**STAT3 as a master switch in cancer-induced DC suppression & MDSC expansion**

Most of the tumor-derived suppressive factors inducing disturbed myeloid development trigger signaling pathways in DC and MDSC, which converge at the level of the transcription factor STAT3. Signal Transducers and Activators of Transcription (STAT) proteins are signaling molecules that are involved in cell survival, proliferation, differentiation and apoptosis, and STAT3 signaling is frequently hyperactivated in many types of cancer\textsuperscript{96}. Following ligand binding, STAT proteins become phosphorylated (i.e. activated), which leads to their homodimerization and subsequent translocation to the nucleus, where they regulate the transcription of target genes. Early hematopoiesis requires JAK2/STAT3 signaling, as this is one of the main pathways used by cytokines that support myeloid cell differentiation\textsuperscript{97}. During normal DC differentiation, however, STAT3 activity is decreased\textsuperscript{98}. This decrease in STAT3 activity in DC was prevented when precursor cells were differentiated in tumor-conditioned media. Inhibition of STAT3 activity abrogated the suppressive effects of tumor-conditioned media on DC differentiation\textsuperscript{98-100}. Similarly, studies have shown that MDSC from tumor-bearing mice have increased levels of phosphorylated STAT3 (pSTAT3) compared with MDSC from naive mice\textsuperscript{101}. Blocking STAT3 expression through the use of conditional knockout mice, or the use of STAT3 inhibitors, led to a reduction in MDSC expansion and an increase in effector T cell responses in tumor-bearing mice\textsuperscript{102,103}. Thus, STAT3 hyperactivation leads to a blockade in IMC-to-DC differentiation and thereby promotes the accumulation of MDSC. Most of the studies indicating the importance of the JAK2/STAT3 pathway in tumor-induced immune suppression, designating it a ‘master switch’, were carried out in mice. Poschke \textit{et al.} did demonstrate overexpression of pSTAT3 in human CD14\textsuperscript{+}HLA-DR\textsuperscript{lo} MDSC\textsuperscript{28}, confirming a possible role for STAT3 signaling in the cancer related accumulation of MDSC. We investigated the pSTAT3 dependence of glioblastoma-
induced inhibition of human DC differentiation in vitro, but were unable to demonstrate a role for the JAK2/STAT3 axis \(^{87}\), nor could we find a relationship between nuclear pSTAT3 expression and infiltration rate of immature DC in non-small-cell lung tumors in an immunohistochemical study \(^{104}\). These studies leave the role of STAT3 signaling in cancer-related suppression of human DC differentiation in doubt, and warrant further investigation of other involved signaling pathways.

**Other DC-related signaling pathways**

PI3K/Akt: The serine/threonine kinase Akt (protein kinase B or PKB) is well known for its critical regulatory role in diverse cellular processes, including cancer progression and insulin metabolism \(^{105}\). The Akt cascade is activated by receptor tyrosine kinases, integrins, B and T cell receptors, cytokine receptors, G-protein-coupled receptors and other stimuli that induce the production of phosphatidylinositol 3,4,5 triphosphates by phosphoinositide 3-kinase (PI3K). These lipids serve as plasma membrane docking sites for Akt and its upstream activator PDK1. There are three known isoforms of Akt kinase, i.e. Akt1, Akt2 and Akt3. Akt1 is activated during pro-inflammatory stimulation of DC and plays a major role in their cytoskeleton motility and survival \(^{106}\). Defective Akt signalling has been reported to result in decreased phagocytosis and migration of DC \(^{106}\). Moreover, DC differentiation from monocytes is accompanied by activation of the PI3K/Akt signaling axis \(^{107}\). Cancer-derived IL-10 effectively inhibits DC functionality through suppression of IκB kinase (IKK) activity and subsequent NF-κB activation; this inhibition of NF-κB activation was reported in part to depend on IL-10 targeting the PI3K pathway \(^{108}\).

**Glycogen synthase kinase-3β (GSK3β)**

GSK3β is a serine/threonine kinase and a key regulator of a wide spectrum of cellular processes, including glycogen metabolism, transcription, translation, cytoskeletal regulation, intra-vesicular transport, cell cycle progression and apoptosis \(^{109}\). It is best known for its ability to regulate Wnt signaling through phosphorylation of β-Catenin \(^{110}\). GSK3β is involved on many levels in inflammatory processes \(^{111}\). Rodionova et al. have reported GSK3β activity to be essential for differentiation of monocytes into DC \(^{112}\). If GSK3β is inactive during early stages of DC differentiation, monocytes will rather differentiate into macrophage-like cells. In immature moDC, inhibition of GSK3β blocked spontaneous maturation of MoDC \(^{113}\). It has been shown that many kinases including protein kinase C, p90RSK and Akt can phosphorylate GSK3β and thereby inactivate it in vitro. However, Akt appears to be the kinase primarily responsible for the phosphorylation of GSK3β in vivo \(^{114}\) and may thereby also modulate DC development.

**p38-MAPK**

An ever-growing body of data demonstrates the importance of the p38 mitogen-activated protein kinase (MAPK) pathway in the cellular response to inflammatory stimuli. The p38 pathway is activated in DC in response to cytokines including TNFα and TGFβ, oxidative stress and TLR-L \(^{115}\). Activation of the pathway results in phosphorylation of MEKKs, MEKs and p38 MAPK in sequence. In turn, p38 phosphorylates downstream kinases such MAPKAPK5, which directly
activate several transcription factors. In a study by Xie et al., it was demonstrated that the differentiation of monocytes into immature DC was accompanied by activation of the Raf/MEK/ERK and PI3K/AKT signaling pathways, whereas p38 MAPK was identified as a negative regulator of DC differentiation. Wang et al. demonstrated that MoDC generated from myeloma patients were phenotypically and functionally defective as a result of high production of IL-6 and that blocking p38 in the progenitor cells could partly overcome this. Indeed, we showed that p38 inhibition could alleviate the suppressive effects on DC differentiation of soluble factors derived from a wide range of solid tumor types. Inhibition of p38 on the whole had a more profound influence than STAT3 inhibition in this regard. This apparent dominance of p38 over STAT3 inhibition in countering DC suppression was confirmed in short-term cultures of primary melanoma suspensions. Combined STAT3 and p38 interference also had a profound effect on the T cell priming capacity of tumor-modulated DC, resulting in Th1-skewed responses. In line with this, decreased release of PGE2 and IL-10 and increased release of IL-12 by p38-inhibited DC was reported to result in effective Th1 skewing.

**Therapeutic intervention: tipping the balance in favor of DC development and anti-tumor immunity**

Clinical experience with tumor vaccines has made it clear that cancer immunotherapy can only be successful if the immune system is operating at optimal efficiency. Indeed, a consensus is emerging that for cancer immunotherapy to be effective, it will have to be applied together with therapies aiming to eliminate tumor-related immune suppression. Myeloid cells, and MDSC in particular, are an obvious therapeutic target in this context. The following paragraphs will summarize different strategies that are being explored to inhibit or eliminate myeloid-induced suppression and redress the tumor-influenced balance in favor of DC development, activation, and effective anti-tumor T cell immunity.

One of the most promising approaches to target immunosuppressive MDSC is to promote their differentiation into more mature myeloid cells that do not have suppressive functions, but may exert anti-tumor effects. Vitamin A metabolites in particular stimulate the differentiation of myeloid progenitor cells into DC and macrophages. Administration of all-trans-retinoic acid (ATRA) resulted in a decrease in MDSC numbers in tumor-bearing mice and cancer patients. ATRA was shown to induce the differentiation of MDSC into DC and macrophages. Decreasing the number of MDSC resulted in increased tumor-specific T cell responses, and the combination of ATRA and a cancer vaccine prolonged the anti-tumor effect in two different mouse tumor models. Recently, the effects of ATRA administration on MDSC numbers was also tested in metastatic RCC patients, resulting in a reduction of peripheral blood MDSC frequencies and enhanced tumor-specific T cell responses. Similarly, early exposure of tumor stromal cells to a high dose of the myeloid differentiation factor, IL-4, was recently shown to prevent the generation of MDSC and to promote T cell mediated tumor rejection.

Other strategies to inhibit the accumulation and expansion of MDSC have focused on neutralizing the effects of tumor-derived regulatory factors. Inhibition of Matrix metallopeptidase 9 (MMP9) in tumor-bearing mice led to decreased MDSC frequencies in spleen and tumor...
tissue, resulting in delayed tumor growth and improved efficacy of tumor vaccination. In addition, inhibition of MMP9 through the administration of amino-bisphosphonates reduced systemic MDSC expansion, as well as the number of TAMs, and decreased VEGF serum levels and impaired tumor growth. Inhibition of SCF-induced signaling through the blockade of its receptor also decreased MDSC expansion and tumor angiogenesis, as did inhibition of VEGF, another tumor-derived and pro-angiogenic factor. Indeed, many of these targeted factors are responsible for promoting angiogenesis, as well as skewing myeloid differentiation to a more suppressive profile. As a result, a number of established or experimental antiangiogenic therapeutics can also be applied to normalize myeloid differentiation in cancer patients and abrogate immunosuppressive effects. Early evidence has already shown that antibody-mediated neutralization of VEGF not only blocked tumor angiogenesis, but was also capable of improving the function of DC in tumor-bearing mice. Furthermore, selective inhibition of VEGF, through blocking of VEGF receptor-2 (VEGFR2) with the monoclonal antibody r84, resulted in decreased MDSC infiltration and increased mature DC infiltration in mice bearing human breast cancer xenografts.

Another example of an established anti-angiogenic drug with beneficial effects on myeloid differentiation is sunitinib. Sunitinib is an oral TKI that has blocking effects on several growth factor receptors, including c-KIT, VEGFR2, PDGF receptor and Flt3. It has been approved by the US FDA as first-line treatment for metastatic RCC. Importantly, besides its obvious anti-angiogenic effects, sunitinib also inhibits the activity of STAT3 in RCC cells, as well as in MDSC and macrophages, and reduces their number in tumor-bearing mice. Sunitinib-induced STAT3 inhibition is correlated with a down-regulation of several STAT3-regulated angiogenic genes that may also negatively influence myeloid maturation, including VEGF. Ozao-Choy et al. demonstrated that tumor-bearing mice treated with sunitinib had decreased levels of MDSC and Tregs in their BM, spleen and tumor, and that the suppressive activity of MDSC was reduced. As a result, IL-10 and TGFβ levels decreased, T cell anergy was prevented and Th1 and CTL responses were enhanced. These findings were confirmed by others in a mammary tumor model. We found evidence for a normalization of myeloid differentiation in RCC patients treated with sunitinib. Treatment with sunitinib for 4 weeks followed by a 2-week-off period resulted in reduced systemic levels of granulocytes, monocytes and MDSC, while circulating DC frequencies increased to levels observed in healthy donors. In particular, higher levels of the BDCA1/CD1c+ cDC subset correlated with clinical tumor responses, and was associated with improved overall survival. MDSC levels in the blood were also shown to decline in RCC patients after treatment with sunitinib, as demonstrated by Ko et al. This decline in MDSC was associated with an increase in IFNy-producing T cells and plasma IFNy levels, and decreased Treg rates. Recently, Ko et al. showed that GM-CSF in the tumor microenvironment activated STAT5a in MDSC, thereby rendering them resistant to the inhibitory effects of sunitinib. This indicates that additional measures may be needed to achieve intratumoral inhibition of MDSC by TKIs.
The above described methods are mostly geared towards restoring the balance in myeloid subsets in cancer patients through systemic intervention. An attractive approach to the induction of specific anti-tumor immunity is through *in vivo* targeting of DC. A particular challenge in this regard is the need to overcome immune suppression and ensure proper DC activation to achieve the induction of an effective and long-term anti-tumor T cell response. As the skin is often the route of choice for vaccine administration, in this thesis we explored the frequency and activation status of DC subsets in the skin microenvironment and its draining LNs as well as ways to target immune stimulatory subsets and overcome local tumor-related immune suppression.

**Targeting DC in vivo: the skin as a preferred site for tumor vaccination**

Numerous clinical trials have been carried out to study the effect of DC-based vaccination with specific TAA. A common strategy is the *ex vivo* generation of autologous DC, which are then loaded with TAA proteins, mRNA or TAA-derived peptides, carrying known CTL and/or Th epitopes, and subsequently re-administered to the patient. With our rapidly growing knowledge of DC biology, it has now become possible to specifically target vaccines to DC *in vivo* and at the same time achieve DC activation. Direct *in vivo* administration of DC-targeted vaccines may present a more attractive and standardized alternative to classic DC-based melanoma vaccination, by-passing the need for costly, time-consuming, and laborious approaches involving the generation and loading of autologous DC *ex vivo*. A dense network of easily accessible and functionally specialized DC subsets lines the skin and can rapidly initiate immune activation upon maturation induction by endogenous or exogenous danger signals. With ready access to afferent lymphatic vessels they can efficiently migrate to draining LNs where they specifically activate T cells (see Figure 4). This makes the skin a preferred site for administration of DC-targeted vaccines. In recent years, the different DC subsets residing in human skin and in draining LNs, and their specialized functions in T cell activation, have started to be unraveled. Most of our knowledge of skin DC subsets derives from mouse studies. In human skin, functional studies have been hampered by low DC numbers and by the inability to deplete them in a targeted fashion. An important issue that as yet is not fully resolved is whether the observed sub-populations among migrated human DC are genuine subsets, or whether they represent the same DC subset in various states of activation or differentiation. A growing number of studies now point to the existence of an inter-related population of cutaneous cDC and macrophages, trans-differentiating into each other as directed by environmental cues. Below, a brief overview is given of known cDC subsets in skin and skin-draining LNs.
**Langerhans Cells (LC)**

LC are the DC of the epidermis and are derived from precursor cells residing in the skin, whereas under inflammatory conditions they can also develop from monocytes recruited from the blood \[^{140,141}\]. They express high levels of CD1a and Langerin at their cell surface, as well as the epithelial adhesion molecule EpCAM. There is evidence to suggest that upon their activation and migration to LNs, LC preferentially bind and activate T cells \[^{142}\]. In keeping with this, LC were shown to be superior CTL activators through their release of IL-15 \[^{142-144}\]. As cell-mediated immunity is generally believed to be crucial in tumor eradication, it may therefore be beneficial to specifically target LC for tumor immunotherapy. LC targeting usually involves trans-cutaneous approaches \[^{145-147}\]. However, there are ways to attract LC to the dermis, where they can also be targeted for immunization purposes. Moreover, a recent study showed that i.d. injected antibodies were able to pass the basal membrane and bind LC in the epidermis \[^{148,149}\].
suggesting that even dermal delivery of large proteins can result in targeting of epidermal LC.

**CD1a⁺ DDC**
CD1a⁺ DDC do not express Langerin and only intermediate to low levels of CD1a. Instead, DDC can express an alternative set of Lectins, including the Mannose Receptor (MR) and DC-SIGN, as well as Factor XIIa \[^{150}\]. The differential expression pattern of antigen capture receptors between LC and DDC should enable the specific targeting of each subset for vaccination purposes. DDC have been shown to produce IL-10 and to be able to direct the generation of type-2 humoral responses, vital to the initiation of humoral immunity \[^{150,151}\]. However, the T cell skewing abilities of interstitial DC/DDC are not fixed, but rather dictated by a balance of factors in the microenvironment, their number, and activation state, resulting in differential Th1, Th2, or Th17 profiles \[^{150,152,153}\]. The ability to modulate this balance may be of crucial importance for successful immunotherapy of cancer. Langerin⁺ and CD103⁺ DDC were identified in murine studies as a major migratory DC subset from skin with the ability to cross-present proteins from the skin environment \[^{154-156}\]. It has been suggested that CD1a⁺ DDC may be the human equivalent of this subset \[^{142}\], but evidence to back up this claim is lacking. We have performed a genome-wide transcriptional profiling analysis of freshly isolated human CD1a⁺ DDC vs. LC and found DDC to express a far wider range of adhesion and co-stimulatory molecules, chemokines, and cytokines (and at higher levels), pointing to a putatively superior migratory and T cell stimulatory ability over LC \[^{157}\]. Indeed, our own comparative *ex vivo* study of the ability of these subsets to prime CD8⁺ effector T cells against a MART-1 epitope, are in keeping with these transcriptional analyses \[^{158}\].

**CD14⁺ DDC**
CD14⁺ DDC and dermal macrophages: Several reports have pointed to the existence of a CD1a⁻ CD14⁺ DDC subset in human skin under steady state conditions with an immature macrophage-like phenotype and lacking T cell priming ability \[^{142,144,159-162}\]. These CD14⁺ DC appeared “immunologically silent” (i.e. lacking co-stimulatory signals and the LN-homing chemokine receptor CCR7) and were further characterized by expression of the C-type lectin BDCA3 \[^{160}\]. Beside CD14⁺ DDC, also CD14⁺ resident macrophages were found in the steady state dermis. These macrophages were strongly positive for the macrophage markers CD68 and CD163 \[^{163}\] but surprisingly have also been reported to express the generally with DC associated C-type Lectin DC-SIGN \[^{164}\]. CD14⁺ dermal macrophages can be discerned from CD14⁺ DDC through their lack of CD1b or CD1c \[^{162,163}\]. While, like CD14⁺ DDC, dermal macrophages display a poor ability to induce T cell proliferation, they may nevertheless contribute to T cell activation through their release of inflammatory cytokines \[^{163}\].

**DC subsets in skin-draining LNs**
As dermally delivered substances will also rapidly diffuse to skin-draining LN, DC subsets residing in these LNs may be directly modulated and/or targeted. Until recently, very little was known about cDC subsets present in human LNs that drain the skin. From our own melanoma
Sentinel LN (SLN) studies we can discern at least three different cDC populations.  

1. **CD1a⁺ DC**: these DC most likely comprise skin-emigrated LC and DDC and express high levels of co-stimulatory molecules, CD83, and CCR7. i.d. administration of GM-CSF leads to further upregulation of their co-stimulatory machinery and CD83 and to increased numbers of these DC in the paracortical LN areas. A high and significant correlation was found between densities of CD1a⁺ DC in the papillary dermis and matching SLNs, strongly suggesting the CD1a⁺ DC subsets in the SLNs to derive from dermis-emigrating CD1a⁺ LC and DDC. Frequencies of these mature CD1a⁺ DC in the SLN also correlated significantly with melanoma-specific CD8⁺ effector T cells, indicative of the validity of this DC subset for tumor vaccine targeting.  

2. **CD11c⁺CD14⁻ DC**: these DC do not express CD1a, but do express CD83 and co-stimulatory molecules on their surface, albeit at lower levels than the CD1a⁺ DC. We found the frequencies of this DC subset to be up-regulated in melanoma SLN upon CpG administration. The functional abilities of this novel subset remain to be established, although expression of TRAIL on their surface suggests a direct cytolytic ability. Expression of the C-type Lectin BDCA3/CD141 on at least part of these DC suggests that the BDCA3⁺ cDC subset in peripheral blood may be their direct precursor, but this remains to be established. Interestingly, a genome-wide transcriptional profiling study suggested BDCA3⁺ DC to be the human equivalent of the CD8α⁺ DC subset in murine spleen, which is known to be the subset with cross-priming and powerful CTL priming abilities. Indeed, in vitro studies have provided evidence for the cross-priming abilities of human BDCA3⁺ DC and thus confirmed this hypothesis.  

3. **CD11c⁺CD14⁺ DC**: although at least part of these most likely represent monocytes or macrophages, low CD83 expression on a sub-population seems to suggest a semi-mature DC phenotype. These might derive from migratory CD14⁺ DDC, but this is not consistent with our observations from skin explant studies that migratory CD14⁺ DDC are immature and do not express CD83. Thus, their origins and function for the moment remain obscure.  

It remains mostly unclear how tumor-induced immune suppression affects the above listed DC subsets and which subsets can best be targeted for efficient induction of anti-tumor T cell immunity. These are the two central questions addressed by this thesis.  

**Outline of this thesis: targeting skin associated DC subsets and conferring resistance to tumor-related immune suppression**  

This thesis in divided into three major sections in which studies are described that 1) explore the effects of tumor-related immune suppression on DC subsets and their activation and functionality in the human skin microenvironment, 2) characterize DC subsets in human skin...
and draining LNs and explore ways in which to target them by adenoviral vaccines, and 3) explore the potential of signalling interference to induce resistance to tumor-related immune suppression in targeted human DC.

I. Tumor-related DC suppression in the skin environment
In chapter 2 we report on extensive phenotypic analyses through which we have identified at least six different cDC subsets among cells migrated from cultured human skin explants. Moreover, we have studied the effects of tumor-related suppressive factors on these subsets and have identified IL-10 as a suppressive cytokine with the unique ability to induce predominant migration of an immature CD14+CD141+DC-SIGN+ DC subset with low levels of co-stimulatory molecules, up-regulated expression of the co-inhibitory molecule PD-L1 and the M2-associated macrophage marker CD163. A similarly immature subset composition was observed for DC migrating from explants taken from skin overlying breast tumors. Of note, this predominant migration of immature CD14+ DDC was accompanied by increased release of IL-10, poor expansion of CD4+ and CD8+ T cells, and skewing of Th responses to favor coordinated FoxP3 and IL-10 expression and Treg differentiation and outgrowth.

Autologous tumor cell-based vaccines may afford protection against unique neo-epitopes derived from individual tumors’ mutanome signatures. In chapter 3 we therefore explored the effects of soluble factors derived from primary colon carcinoma on DC migrating from human explant cultures as well as on the maturation of monocyte-derived DC (MoDC). We show that in both instances maturation is enhanced with increased levels of co-stimulatory molecules leading to an enhanced ability for T cell priming. In MoDC this effect was demonstrably prostaglandin-mediated but not so in skin-derived DC. Importantly, this maturation induction was accompanied by a reduced IL-12p70 release by skin-derived DC and a transcriptional signature consistent with Th17/Th22 induction. Possible consequences for whole-tumor cell based vaccine formulations will be considered.

II. Targeting DC subsets in the skin and skin-draining lymph nodes
In chapter 4 we describe the phenotypic characteristics of four cDC subsets present in the first-line draining LNs, i.e. sentinel LNs (SLNs), from early-stage melanoma patients and test their relative ability for T cell activation. Our data point to the possibility of targeting LN-resident DC subsets for anti-tumor vaccination. In chapter 5 we report on the utility of a panel of modified adenoviral vectors for the targeting of DC and show that an Ad5/3 knob-swab variant is particularly suited to selectively target mature DC subsets in skin and melanoma-draining SLN, making it an attractive tumor vaccination vehicle.

III. Targeted signaling interference to induce resistance to tumor-related suppression in DC
In chapter 6 we describe the effects of IL-10 on MoDC maturation. We observed the de novo trans-differentiation of a CD14+ population with a low T cell stimulatory capacity and pro-angiogenic and pro-invasive transcriptional profile. This trans-differentiation event was
prevented by STAT3 mRNA knock-down prior to IL-10 driven DC maturation. In the studies described in chapter 7, we employed a functional kinomics approach to explore signaling cascades involved in tumor-induced DC suppression. GSK3β was identified as a key regulator of DC development and suppression. Moreover, transduction with an adenoviral vector encoding constitutively active GSK3β conferred resistance on DC against the suppressive effects of IL-10.

In chapter 8, the summarizing discussion, the main findings from these studies will be discussed in relation to the latest insights and state-of-the-art in the field.
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