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Allogeneic dendritic cells as adjuvants in cancer immunotherapy

GRAMMATIKI FOTAKI

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Abstract
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In recent years, immunotherapeutic approaches have achieved remarkable successes through checkpoint blockade antibodies, advances in the use of chimeric antigen receptor (CAR) T cells and new insights into the immunosuppressive role of the tumor microenvironment (TME). Through the advances, the role of cancer vaccines based on *ex vivo* manipulated autologous dendritic cells (DC) has been challenged. The main aim of DC-based vaccination is the induction of tumor-specific T-cell responses through presentation of tumor-associated antigens. However, this process has been found to be highly dependent on the ability of the injected vaccine-DCs to activate endogenous bystander DCs.

In this work, we examined the feasibility of having an allogeneic source of vaccine-DCs (alloDCs), not for direct antigen-presentation to T cells but as an immune primer aiming to activate bystander DCs. In paper I, we treated alloDCs with a T helper cell type 1 (Th1)-promoting maturation cocktail alone or combined with a replication-deficient, infection-enhanced adenoviral vector (Ad5M) as a potential gene delivery vehicle. We found that mature pro-inflammatory alloDCs, either non-transduced or transduced, created a cytokine- and chemokine-enriched milieu *in vitro*, and promoted the activation of co-cultured immune cells, including cytolytic NK cells, from unrelated donors. The emerged milieu induced the maturation of bystander DCs, which cross-presented antigens from their environment to autologous antigen-specific T cells. In paper II, we found that alloDCs promoted the migration of murine immune cells both to the site of injection and to the draining lymph node. When Ad5M was used for the delivery of the melanoma-associated antigen gp100, we found that gp100-expressing alloDCs were able to control tumor growth through gp100-specific T-cell responses and alteration of the TME. In paper III, we found that co-administration of alloDCs with an adenoviral vector encoding for HPV-antigens is effective in controlling the growth of HPV-related tumors and this may depend on a cross-talk between alloDCs and NK cells which leads to further recruitment of immune cells into the TME. In paper IV, we observed that concomitant targeting of immune checkpoint receptors or co-stimulatory molecules results in synergistic therapeutic effects in a murine colorectal model.

Keywords: Allogeneic dendritic cells, immune primer, adjuvant, adenoviral vector, cancer immunotherapy, tumor microenvironment

Grammatiki Fotaki, Department of Immunology, Genetics and Pathology, Clinical Immunology, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

"We're supposed to help fix things.
That's what all of us are.
Tools to fix the broken universe.
There are problems in reality.
You're supposed to repair them.
Each of us has a different purpose."

- Francis to Dirk, Dirk Gently's Holistic Detective Agency

List of Papers

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

I   **Fotaki G**, Jin C, Ramachandran M, Kerzeli IK, Karlsson-Parra A, Yu D, Essand M. Pro-inflammatory allogeneic DCs promote activation of bystander immune cells and thereby license antigen-specific T-cell responses. Oncoimmunology (2018) 7(3):e1395126.

II **Fotaki G**[†], Jin C[†], Kerzeli IK, Ramachandran M, Martikainen MM, Karlsson-Parra A, Yu D[*], Essand M[*]. Cancer vaccine based on a combination of an infection-enhanced adenoviral vector and pro-inflammatory allogeneic DCs leads to sustained antigen-specific immune responses in three melanoma models. Oncoimmunology (2018) 7(3):e1397250.

III **Fotaki G**[†], Ma J[†], Jin C, Karlsson-Parra A, Yu D[*], Essand M[*]. Therapeutic vaccination of HPV-associated tumors using pro-inflammatory allogeneic dendritic cells and an HPV-E6/E7-encoding vector. *Manuscript*

IV **Fotaki G**, Yu D, Essand M[*], Karlsson-Parra A[*]. Concomitant targeting of PD-1 or CD137 enhances the effect of adjuvant pro-inflammatory allogeneic dendritic cells. *Manuscript*

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† Equal contribution – First author
* Equal contribution – Last author

Other papers by the author

1. Jin C, **Fotaki G**[¶], Ramachandran M[¶], Nilsson B, Essand M, Yu D. Safe engineering of CAR T cells for adoptive cell therapy of cancer using long-term episomal gene transfer. EMBO Mol Med, 2016, 8: 702-71.

2. **Fotaki G**, Yu D. Allogeneic dendritic cells in cancer immuno-therapy: a clinical and experimental perspective. J Immuno Immunother. 2017, 1:1.

¶ Equal contribution – second author

Contents

Abbreviations

ALL	Acute Lymphoblastic Leukemia
alloDC	Allogeneic dendritic cell
APC	Antigen presenting cell
Bcl-2	B-cell lymphoma-2
BCR	B cell receptor
BM	Bone marrow
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CEACAM1	Carcinoembryonic antigen cell adhesion molecule 1
CI	Checkpoint Inhibitor
CRS	Cytokine release syndrome
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3-grabbing non-integrin
DLBLC	Diffuse large B-cell lymphoma
dMMR	Miss-match repair deficiency
ECM	Extracellular matrix
EMA	European Medicine Agency
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
Flt3L	FMS-like tyrosine kinase 3 ligand
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
LAG-3	Lymphocyte activation gene 3
LC	Langerhans cell
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
M1	Type-1 macrophages

MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MP	Mononuclear phagocyte
MSC	Mesenchymal stem cell
MSI-H	High micro-satellite instability
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death 1
pDC	Plasmacytoid dendritic cell
PGE2	Prostaglandin 2
PI3Kγ	Phosphoinositide 3-kinase gamma
PMBLC	Primary mediastinal large B-cell lymphoma
PRR	Pattern recognition receptor
PSA	Prostate-specific antigen
PVR	Poliovirus receptor
scFv	Single chain variable fragment
STING	Stimulator of interferon genes
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIGIT	T Cell Ig and ITIM Domain
TIL	Tumor infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Treg	Regulatory T cell

Introduction

The immune system in a nutshell

The immune system is a highly effective and complex group of defenses against exogenous and endogenous dangers and consists of immune cells and effector molecules. All immune cells are produced in the bone marrow (BM) from pluripotent hematopoietic stem cells that give rise to two major categories of common progenitor stem cells, the myeloid and the lymphoid progenitors. Lymphoid progenitors differentiate mainly into natural killer (NK) cells, T cells and B cells, while the other leukocyte populations, as well as erythrocytes, derive from myeloid progenitors. Primary lymphoid organs, being the locations of immune cell production and differentiation, are the BM and the thymus. Secondary lymphoid organs, where immune cells are educated, expanded and maintained, are the spleen, lymph nodes (LNs), tonsils and Peyer's patches [1]. In brief, shortly after they are produced, immune cells leave the BM or thymus and enter the blood circulation. From there they can either roam the body in search of potent danger signals or move into secondary lymphoid organs and other tissues.

Innate and adaptive arms of the immune system

The speed and the specificity of an immune reaction differentiate the innate from the adaptive immune responses. The innate responses are fast and "non-specific". This is because innate immune cells, namely eosinophils, basophils, neutrophils, macrophages, dendritic cells (DCs), mast cells and NK cells, identify via pattern recognition receptors (PRRs) distinctive pathogen-associated molecular patterns (PAMPs) derived from pathogens or damage-associated molecular patterns (DAMPs) from compromised cells and immediately respond in order to eliminate the problem [2]. Innate responses confer short-term immunity and are generic against subsets of pathogens. Chemokines and cytokines released from injured or infected cells, as well as the complement cascade proteins are additional components of the rapidly responding innate arm of immunity [1].

   The adaptive immune responses are slower because they require education in order to efficiently react against foreign agents, but confer a layer of specificity against each particular malicious agent. The primary adaptive responses require more time to develop, but if the same hazard is met again

the secondary adaptive responses are quicker due to immunologic memory. Memory develops because the educated adaptive immune cells, namely B and T lymphocytes, can persist as a small population of long-lasting and specialized memory cells [1, 3]. B and T cells recognize clues of harmful potentials, called antigens, via specialized receptors and co-ordinate further responses. Antigens form a broad group of molecules including self or foreign (non-self) proteins and environmental particles. B cell receptors (BCRs) are membrane-bound immunoglobulins on the surface of B cells, which allow B cells to directly recognize an antigenic target. Once a target is recognized, B cells mount fragments of the target antigen on their surface so they can receive help signals from CD4$^+$ helper T (Th) cells in order to proliferate and differentiate into an antibody-secreting plasma cell [4]. Antibodies are secreted immunoglobulins with the same specificity as the BCR, binding to a specific target, signaling its elimination. T cell receptors (TCRs) help T cells recognize antigens bound to major histocompatibility complex (MHC) molecules on cells, as we will analyze in the next section. There are two types of T cells: CD4$^+$ Th cells interact with antigen-presenting cells (APCs), such as DCs, macrophages and B cells, via cell-bound molecules and/or secreted cytokines. Th cells interact also with CD8$^+$ T cells, mainly via cytokines, and their goal is to provide help signals in order to support optimal immune responses. CD8$^+$ cytotoxic T (CTLs) cells recognize antigens presented on the surface of cells as part of the patrolling process of the immune system. When CTLs identify antigens on infected cells or tumor cells they can eliminate them by releasing cytotoxins, such as perforin and granzyme-B [1, 5]. While both B and T cells will proliferate upon activation, exert their functions and die, some of their offspring will remain as memory cells, aiding the organism to detect faster and confront more efficiently future invasions from the same pathogen.

Presentation of self and non-self

As mentioned above, TCRs differ from BCRs in that they cannot bind to an antigen directly, but only when the antigen is processed and presented as a unique combination of a short peptide fragment bound to an MHC, or else called human leukocyte antigen (HLA), molecule on the surface of cells. Conversion of antigens into MHC-bound peptides is a critical procedure involved in the establishment and maintenance of both tolerant and protective T cell responses. The main types of MHCs, called MHC class I and MHC class II, are distributed differently among cells, interact with different TCR complexes and are involved in distinct antigen-presentation mechanisms [1, 6].

MHC class I molecules are present on the surface of all nucleated cells and interact with the TCR of CD8$^+$ T cells. A functional peptide/MHC class I molecule is assembled in the endoplasmic reticulum (ER), where a peptide

8-10 amino acids long is acquired from the cell's endogenous antigenic pool and inserted in the peptide-binding groove of the MHC class I molecule, before it's translocated to the cell membrane. The MHC class I peptidome consists of proteasome-degraded self-proteins and, in the case of infection, viral products. This endogenous peptide pool also consists of all the defective products of translation and is affected by external stimuli able to dysregulate parts of the process and thus the consistency of this peptide pool [7, 8]. For example, pro-inflammatory cytokines have been found to control the expression of specific subunits of the immuno-proteasome that produces peptides which bind stronger on MHC class I complexes [8]. These events are particularly important in the cancer setting, as they can promote the presentation of already mutated peptides that are entirely absent during the thymic self-restricted T-cell tolerance, termed neoantigens, and can be detected by tumor-specific CTLs [9].

MHC class II molecules, which interact with CD4^{+} T cells, are primarily expressed on professional APCs, namely DCs, macrophages and B cells. Endothelial cells, granulocytes and activated T cells, among others, have also been found to express MHC class II molecules, too, though only upon inflammatory stimuli and under specific conditions [10, 11]. The peptide pool of MHC class II molecules derives mainly from exogenous proteins degraded in the endosomal compartment into 15-24 amino acid long peptides. MHC class II molecules assemble in the ER and translocate to the MHC class II compartment of a late endosome, where a specific peptide will complete the peptide/MHC class II complex before it is transported to the cell membrane.

One important aspect of the MHC class II presentation is that it can be intercrossed with that of MHC class I, leading in antigenic fragments from exogenous proteins being presented via MHC class I molecules to CD8^{+} T cells, a process called cross-presentation. Cross-presentation can occur either via ingested antigens entering the cytosol and follow the typical MHC class I loading process [12, 13] or via the simultaneous presence of candidate peptides and MHC class I molecules in the endosomal compartment [14-16]. This process is important in cancer elimination, as it allows for APCs, particularly cross-presenting DCs, to acquire neoantigens or tumor-associated antigens (TAAs) from destroyed cancer cells and sensitize CTLs against them [17].

A plexus of scavengers, communicators and effectors

Upon discovering a danger, innate immune cells will secrete cytokines and chemokines in order to alert the immune system of a potentially hazardous situation. Particularly important is the fact that in response to PRR-ligands and/or inflammatory cytokines DCs (communicators) become activated and

migrate to LNs where they present antigens collected on-site to T cells (effectors) initiating this way a complete immune reaction [18]. We will look further into this particular interaction as it plays a major role in cancer immunotherapeutic approaches; including the one my thesis is based on.

Dendritic cells

In 1973, Steinman and Cohn were the first to describe a type of innate immune cell able to assume a variety of branching forms, resembling a branched tree [19]. Due to this resemblance these cells were termed as dendritic cells after the Greek word for tree, dendreon. These peculiar cells were initially thought to originate from myeloid progenitors, however it was later found that DCs can arise from lymphoid progenitors, too [20]. DCs are professional APCs and the key linkers of the innate and adaptive arms of immunity. They reside in most tissues, functioning as immune sentinels for invading pathogens or damage. They process antigenic information from the peripheral tissues and mount them on their surface, following traffic to secondary lymphoid organs to orchestrate adaptive immune responses [21].

DCs in vivo and in vitro

In human and mice four main subsets of DCs can be identified: the myeloid or conventional DCs (cDCs), the plasmacytoid DCs (pDCs), the Langerhans cells (LCs) and the monocyte-related DCs [22]. Different subsets of inflammatory, monocyte-derived or non-classical DCs have been described, highlighting the complexity of the DC subtypes and how their functionality is linked to their location and local microenvironment [23-25]. In human, two distinct cDC populations have been identified based on the expression of CD1c and CD141 [26], though both populations can be present in lymphoid and non-lymphoid tissues. Murine lymphoid tissue cDC subsets can be separated based on the expression of CD8α and CD4, while migratory, non-lymphoid tissue cDC subsets based on CD11b and CD103 [20]. Recent findings indicate that CD141^{+} human DCs are counterparts for CD8α^{+}/CD103^{+} murine DCs, while human CD1c^{+} DCs resemble the CD11b^{+} murine subset [27, 28]. The reality of these remarkable cells is far more puzzling though, since identification markers can be shared with other cells of the mononuclear phagocyte (MP) system or bear no relation between human and murine subsets [22, 25, 26].

In vitro, human DCs can be obtained through the isolation and differentiation of CD34^{+} hematopoietic progenitor cells [29] or monocytes from peripheral blood mononuclear cells (PBMCs) [30, 31]. While CD34^{+} progenitors are very rare in blood, monocytes can be enriched after leukapheresis resulting in the broad use of the PBMC-monocyte method for *in vitro* generation of DCs. Within the last two decades, different protocols utilizing sets

of cytokines have been developed in order to differentiate monocytes into certain DC populations that differ in their homogeneity and immunomodulatory activity [32]. The most extensively used protocol is based on the use of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) and results in immature DCs with a moderate expression of MHC class I and II, co-stimulatory molecules CD80 and CD86, CD40, but not CD14 or CD83 [30, 31]. Other protocols for example, involve the use of GM-CSF with interferon-alpha (IFN-α) [33, 34], tumor necrosis factor-alpha (TNF-α) [35] or IL-15 [36], all resulting in immature or semi-mature DCs with different capacities for antigen-uptake and T-cell stimulation [32]. Nowadays, commercialized protocols for the isolation and direct use of the most relevant, naturally circulating DCs are proposed to circumvent the need for lengthy differentiation protocols [37]. In all cases, an additional protocol is required in order to achieve fully mature DCs. Several groups have developed successful maturation protocols involving cocktails of cytokines or cytokines and Toll-like receptor (TLR) ligands for this purpose [38-40]. Similar *in vitro* DC generation and maturation protocols exist in the murine system, with BM being the main source of cells and the cytokines GM-CSF and FMS-like tyrosine kinase 3 ligand (Flt3-L) to play crucial roles [41, 42]. Debate regarding the use of GM-CSF over Flt3-L, and *vice versa*, is based on the *in vivo* role of those cytokines, the homogeneity of the generated population and its immunostimulatory capacity [43, 44].

DC maturation and T-cell polarization capacity

In most tissues, DCs exist in an immature state, sensing their environment and taking up antigens, but unable to stimulate any immune cell activation [45]. Immature DCs presenting self-antigens or innocuous environmental clues maintain homeostasis and tolerance either by silencing responding T cells or by expanding regulatory T cells (T_{regs}) [46, 47].

Maturation converts the DCs from antigen-capturing to antigen-presenting cells, decreasing their capacity to collect antigens but increasing their ability to stimulate T-cell responses [45]. Some very well characterized PRRs, namely TLRs, have distinct functions in pathogen and damage recognition [48, 49]. Direct TLR signaling or signaling via inflammatory cytokines initiate DC maturation, which is marked by the upregulation of MHC class I and II molecules, activation markers, co-stimulation molecules and secretory signals. DC maturation is very important in immunological activation of effector T cells, which require simultaneous antigenic sensitization (signal 1), co-stimulation (signal 2) and exposure to cytokines (signal 3). Activation induces the expression of CCR7 which leads to DC migration to the lymph-node via afferent lymphatics in response to CCL19/CCL21 [50]. Along with antigen-recognition signal 2 is transmitted through CD80 and CD86, which interact with CD28, a receptor constitutively expressed on naïve T cells [51] (Figure 1). The activation receptor CD40 is expressed on

DCs and binds to CD40 ligand (CD40L) which is induced on CD4^{+} T cells after antigen recognition [51]. This CD40-CD40L interaction is important for DCs as it licenses them to secrete higher levels of IL-12 which is crucial for optimal CTL responses. It has been found that a variety of DC conditioning stimuli dictate the fate of DC maturation and subsequent T-cell polarization (signal 3), with outcomes varying from immuno-suppressing to immuno-activating roles [52-54]. This is particularly important for the function of Th cells as they communicate an immune response to a plethora of other immune cells, such as CTLs, macrophages and B cells [55].
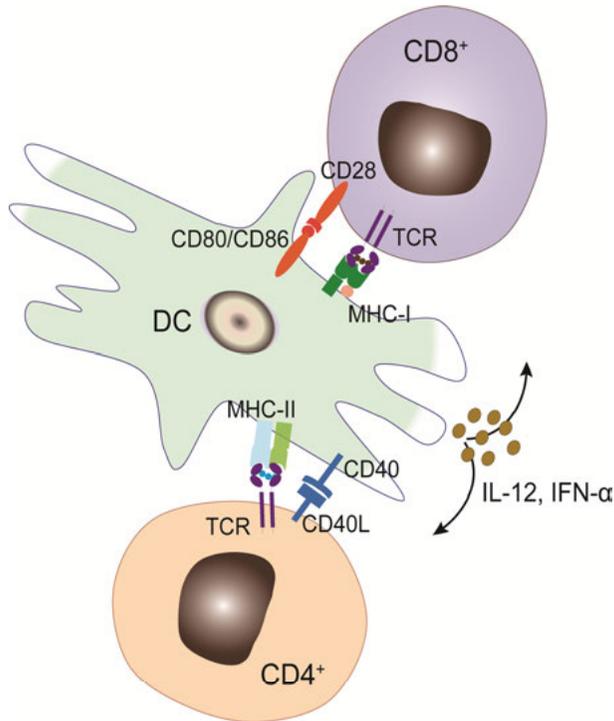
Figure 1. **Cross talk between DCs and T cells.** Priming of T cells by DCs requires interaction between peptide/MHC complexes expressed on DCs and T cell receptors (TCRs) expressed on T cells (signal 1) and co-stimulation, importantly CD80/86 provided by the DCs that is recognized by CD28 on T cells (signal 2). In addition CD4^{+} T cells provide stimulation to DCs through expression of CD40 ligand (CD40L) interacting with CD40 on DCs. DCs further secrete cytokines (signal 3) that contribute to the fate of the T-cell response.

T cells

T-cell development is a two-step process taking place in the thymus, the end product of which is T cells able to recognize self-peptide/MHC complexes without reacting to them [1]. In order to do so, T cells carrying a TCR are tested first on the functionality of their TCR, meaning their ability to bind

self-peptide/MHC complexes (positive selection). On the second level, T cells with functional TCRs are tested on the strength of the TCR recognition and follow elimination when interacting strongly with self-peptide/MHC complexes (negative selection). Naïve T cells with low affinity to self-antigens are leaving the thymus to colonize peripheral lymphoid organs, while T cells that failed negative selection can either go into apoptosis or differentiate into T_{regs}.

T-cell activation and polarization

Naïve T-cell activation is carried out after strong interactions with APCs, particularly DCs, and is antigen-specific. A nanoscale gap between APCs and T cells termed the immunological synapse is fundamental for the activation of T cells [56]. Immunological synapses provide specialized antigenic activation through the peptide-MHC/TCR complex. Though strong, this interaction is further strengthened by the incorporation of the CD4 or CD8 co-receptors binding directly on the non-polymorphic regions of the MHC class II or I, respectively [56, 57]. Adhesion signals are transmitted via the LFA-1/ICAM-1 and CD2/CD58 interactions and co-stimulation signals mainly via the CD28/CD80 or CD86 complexes [56]. Co-stimulatory interactions are very important at this point as antigenic recognition with suboptimal co-stimulation induces anergic, non-responsive T cells [56]. Activated T cells form similar immunological synapses with target cells as well. Via the synapse, CTLs dock on infected or tumor cells and eliminate them by secreting perforin and granzyme [58].

During T-cell priming, the proximal concentration of cytokines secreted by APCs is important in the polarization of the different subsets as it dictates different transcription programs. Differentiation of CD4^{+} Th cells is governed by distinct combinations of cytokines and transcription factors [5, 55, 59]. Th-cell activation in the presence of IL-12 leads to induction of Th1 responses, which are important during cell-mediated immunity [5, 60], while presence of IL-4 dictates the polarization to Th2 responses which play important roles in humoral immunity [5, 61]. Other main Th subsets are the Th17 and the T_{regs} subsets [5, 55, 59]. The combination of transforming growth factor (TGF)-β with IL-6 induces Th17 responses, while T_{regs} are polarized in the presence of TGF-β with IL-10 and IL-2 [5, 55]. One interesting aspect of the T_{regs} subset is that those occurring after negative selection are considered to be parts of the process of central tolerance and are educated to suppress responses against self-antigens [55]. However, T_{regs} induced and polarized in the periphery, for example during tumor-related immune responses, play parts in peripheral tolerance and can provide immunosuppression against TAAs. Cytokines have been found to have a polarization outcome also in CD8^{+} effector T cells, with IL-12 affecting the effector memory phenotype and IFN-α the central memory [62].

Co-inhibitory mechanisms

An uncontrolled immune reaction can be dangerous for the integrity of the organism and can result to autoimmunity. Under inflammation endogenous control circuits have the ability to negatively regulate excessive activation by co-inhibitory receptors. Two of the most known inhibitory interactions are mediated by cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed cell death-1 (PD-1) protein, which are also referred to as immune checkpoint receptors [56, 63]. CTLA-4 is expressed upon T-cell activation and binds CD80/CD86 with higher affinity than CD28. Thus, CTLA-4 signaling impairs CD28-dependent priming either directly via attenuation of TCR signaling cascade in T cells or indirectly by blocking access to CD80/CD86 on APCs [56, 64]. PD-1 is also expressed after TCR-engaged T-cell activation and its ligands, PD-L1 and PD-L2, are upregulated by IFN-γ during inflammation [56, 63]. Binding of PD-1/PD-L1 on primed CTLs dampens TCR signaling through recruitment of tyrosine phosphatases that inhibit TCR-proximal kinases important for T-cell activation [65]. Other studied checkpoint molecules include the lymphocyte-activation gene 3 (LAG-3), the T Cell Ig and ITIM Domain (TIGIT) and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) [63]. Such molecules are upregulated after T-cell activation and mediate inhibition of T-cell responses in similar manners to CTLA-4 and PD-1. LAG-3 binds with high affinity to MHC class II, while TIGIT to poliovirus receptor (PVR) and TIM-3 to galectin-9 and CEACAM1 [63].

The immune system in cancer

Cancer Immunoediting

Cancer development is a multistep process through which normal cells are transformed into malignant cells with abnormal cell growth and the potential to invade and spread to other parts of the body [66]. Loss of normal cellular regulation can lead to the upregulation of antigens that are not presented or presented in low levels on the surface of normal cells, therefore referred to as tumor-associated. Typical examples of these TAAs are the carcinoembryonic antigen or the cancer/testis antigen and are usually present in different groups of tumors. On the other hand, mutations can give rise to variations in protein sequences, resulting in malignant cells expressing an altered protein repertoire than normal cells, referred to as neoantigens. Such unique tumor-distinctive antigens are released when tumor cells die and can be taken up by immature DCs. DCs can mature and migrate to draining lymph nodes, where they can activate tumor-specific CD8^{+} T cells via cross-presentation of captured neoantigens, initiating the control and elimination of the mutated cells. Tumor-specific killing will lead to further release of antigens and the crea-

tion of a pro-inflammatory milieu, which will attract and employ more immune cells on site. This effector "homing-killing" cycle is referred to as the cancer-immunity cycle [67] (Figure 2).
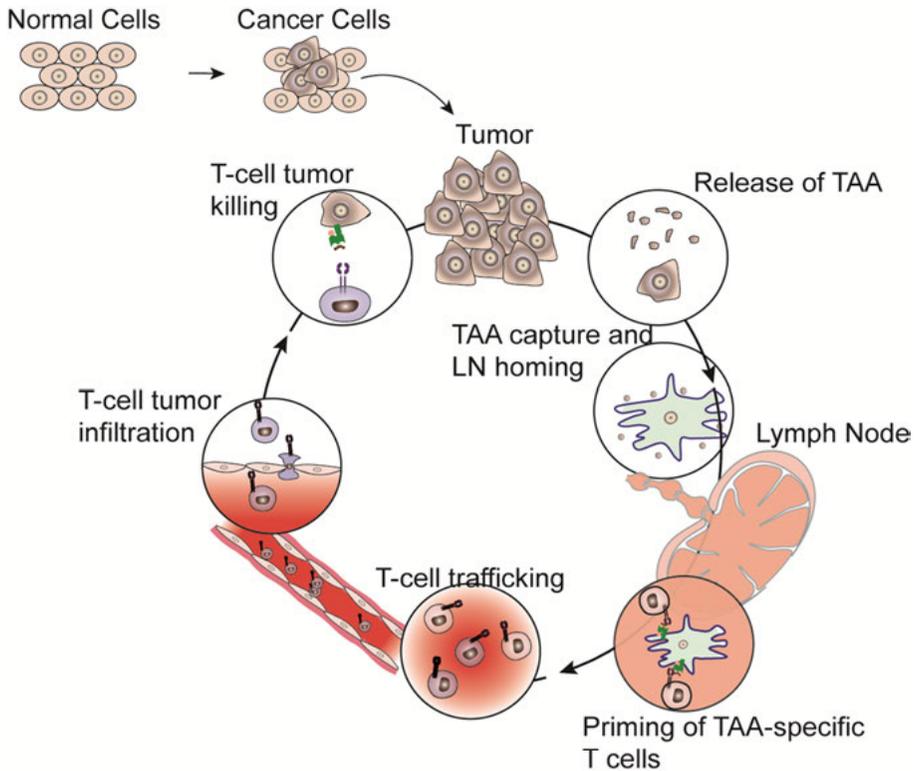
Figure 2. **Cancer immunity cycle.** When tumor cells die, tumor-associated antigens (TAAs) and neoantigens are released into the micro-environment and can subsequently be taken up and cross-presented by antigen presenting cells (APCs) in the draining lymph node (LN). This will mount a T-cell mediated immune response which will result in the further death of tumor cells and the kick-starting of another round of the cycle.

The hallmark of evading this cycle of immune recognition and destruction [66] is in fact a complex interplay between immune cells and tumor cells [68, 69]. Sir McFarlane Burnet in 1957 introduced the tumor immune surveillance concept, which described the ability of the immune system to recognize and eliminate tumors [70]. Through the years this theory was challenged and refined into the cancer immunoediting hypothesis, which occurs in the three phases: elimination, equilibrium and escape [71, 72] (Figure 3). The elimination phase is actually the core of the aforementioned cancer-immunity cycle [73]. Tumor elimination involves a plethora of immuno-activating molecules and interactions, involving both the innate and adaptive immunity [74]. Genetic aberrations lead tumor cells, in addition to express-

ing and presenting tumor antigens, to express stress-induced molecules, as for example NKG2D ligands thereby activating NK cells. Intratumoral DCs can take up tumor antigens, further enhancing CD8^{+} T-cell activation and IFN-γ release, which in turn inhibit tumor cell proliferation and angiogenesis [75, 76]. DNA released from destroyed tumor cells has the potential to induce IFN-α/β via the cytosolic STING-activation pathway that subsequently promotes DC maturation and T-cell stimulation [77]. Other innate immune cells attracted to the site, such as type-1 macrophages (M1) and granulocytes, secrete IFNs, TNF-α, IL-12, IL-1 and other cytokines, contributing further to antitumor immunity [74]. When tumor variants survive the elimination phase they may enter into an equilibrium phase, driven by a balance between acquiring mutations that will affect immunogenicity and immunoevasiveness. Interestingly, immune cells provide this selective pressure that sculpts tumor's evasive features, as they eliminate immunogenic variants. The equilibrium phase has been stated to be a long procedure during which the most immunoevasive mutations can initiate the escape phase and tumor outgrowth [73]. During escape, the cancer-induced immunosuppression is established and leads to a clinical apparent disease [73]. The events between Equilibrium and Escape are much interrelated, shaped in the common axis of avoidance of immune recognition and elimination. This can be achieved through downregulation of MHC class I molecules on tumor cells, upregulation of inhibitory molecules, such as PD-L1, expression of immune-suppressive cytokines or survival factors, such as the IL-10 or the anti-apoptotic molecule Bcl2, and the creation of a tumor-supporting microenvironment [69, 74].

Tumor Microenvironment (TME)

The term TME describes a multifaceted network of all the cells and extracellular components at the tumor site that are not tumor cells, but are recruited throughout the immunoediting stages. These non-tumor components originate mainly from leukocyte and mesenchymal lineages, including cancer-associated fibroblasts (CAFs).

Leukocyte populations have a variable composition among different tumor types and stages. The mere presence of leukocytes in the tumor environment cannot be solely determined as immune suppressing, as they can also have anti-tumor effects [78]. The most recent view on the tumor immune microenvironment dictates that tumors can be categorized as immune-deserted, -excluded or -inflamed, depending on the activation status and the infiltration of immune cells, particularly lymphocytes [79]. Immune-deserted tumors are characterized by a low infiltration of immune cells. Lymphocytes located in the periphery characterize immune-excluded tumors, while immune-inflamed tumors can be identified by high numbers of immune cells infiltrating the tumor core. T cells, macrophages and granulocytes which ha-
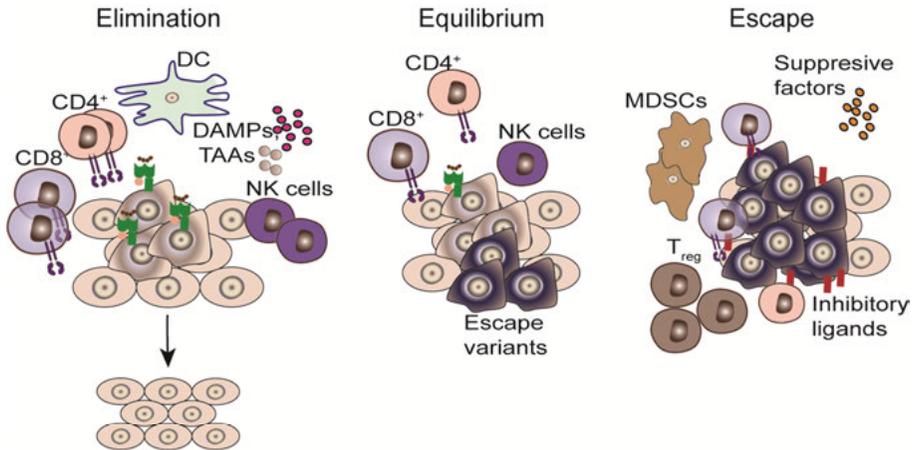
Figure 3. **Cancer Immunoediting.** Cancer immunoediting occurs in three phase: Elimination, Equilibrium and Escape. It's a complex, dynamic process where immune-surveillance and tumor progression events success each other until tumor-induced immunosuppression is evading immunological responses.

ve arrived at the tumor site at later stages and rendered anergic and/or immunosuppressive, such as T_{regs} and tumor-associated macrophages (TAMs), can also be found in the TME [80]. T_{regs} function normally by suppressing the immune responses to self-antigens, a very profitable feature for tumor cells to exploit. T_{regs} can secrete inhibitory cytokines, mediate direct cytolysis and metabolic exhaustion of effector cells and/or inhibit DC function [81]. TAMs are evolved from macrophage progenitors under the pro-tumorigenic environment of the tumor lesions, mainly due to the presence of IL-4, IL-10 and/or TGF-β [80, 82]. Apart from releasing immunosuppressive factors, TAMs also recruit T_{regs} via CCL22 and contribute to neovascularization events [80]. Another important TME-related population composed of immature myeloid cells is described as myeloid-derived suppressor cells (MDSCs) [83]. MDSCs can be divided into monocytic and granulocytic and function mainly through promoting oxidative stress, depleting TME of crucial nutrients and recruiting T_{regs} [80, 83].

Other TME components include mesenchymal stem cells (MSCs), fibroblasts and endothelial cells [80]. CAFs and MSCs have been proposed to support the generation and maintenance of a stem cell niche and in this way favoring tumor growth [84]. Endothelial cells and pericytes in the TME are severely dampened, resulting in tumor-driven effector cells' exclusion, angiogenesis and cancer-cell dissemination [80, 85]. Moreover, the extracellular matrix (ECM) normal functions are to maintain tissue architecture and sense abnormal cell behavior, functions completely abrogated in the TME. A dysfunctional ECM occurs mainly through deregulation of ECM remodeling enzymes by CAFs, MSCs and TAMs [86, 87].

Cancer Immunotherapy

The importance of oncoimmunology

The application of immune-control on tumor cells can be traced back to the 1890s when William Coley tried to awake the immune system against cancer by using bacteria as stimulants [88]. Coley set the groundwork for early cancer immunotherapy approaches, though his attempts had limited success due to limited understanding of the underlying tumor immunology and the complex interactions between tumor cells, immune infiltrates and stroma [89]. In recent years, oncoimmunology has progressed to the point where dissecting cellular composition, localization and functional status of the non-cancer cells provide crucial information on treatment approach and clinical outcomes [78, 90]. Though there is a long road ahead until the majority -if not all- of the patients benefit from immunotherapies, advances in oncoimmunology has helped cancer immunotherapeutic discoveries to become a "Breakthrough of the Year" in 2013 [91] and their significance awarded James P. Allison and Tasuku Honjo the 2018 Nobel Prize in Physiology or Medicine. Most importantly, they have led to the discoveries of new medicines which revolutionized the traditional cancer treatment pillars due to their unprecedented success in advanced disease.

Main Immunotherapeutic approaches

Cancer immunotherapy is the treatment that uses the immune system to help fight cancer. Its most successful forms until now can be summarized in efforts to eradicate tumors by stimulating a TME-exhausted immune system or by isolating, educating and returning patients' immune cells in order to evoke responses against tumor cells. Either indirectly or directly, the ultimate goal is to evoke and maintain highly functional tumoricidal CTLs to control tumor progression and halt metastasis.

Checkpoint inhibitors and co-stimulatory molecules

Checkpoint receptors that normally balance the immune system's homeostasis can be overtaken by tumor cells and blocking them has emerged as one of the major key-players in releasing the TME-induced immune breaks. There are several checkpoint inhibitors (CIs) approved by the United States Food and Drug Administration (FDA) and the European Medicine Agency (EMA). Ipilimumab (Yervoy®) targets CTLA-4, while the PD-1/PD-L1 interaction is targeted by Nivolumab (Opdivo®) or Pembrolizumab (Keytruda®) for PD-1 and by Durvalumab (Imfinzi®), Avelumab (Bavencio®) or Atezolizumab (Tecentriq®) for PD-L1. Alone or in combination, blockade of checkpoint molecules is among the most effective and novel immunotherapeutic approaches for solid tumors, showing great success especially in

advanced melanoma, lung and renal cancer [92-95]. Blockade of PD-1 is also particularly successful in Hodgkin's lymphoma [96], Merkel cell carcinoma [97] and as a tissue-agnostic drug for solid tumors with high microsatellite instability (MSI-H) and/or miss-match repair deficiency (dMMR) [98]. Although CIs are a great advancement in the field, autoimmune-like adverse events affecting the gastrointestinal track, skin, pancreas and kidneys have been strongly associated to CI-treatment [99]. Additional challenges posed by treatment resistance and clinical benefit only to an average of 30% of the patients have lead research to further explore other tumor-abused T cell inhibitory pathways, such as LAG-3 and/or TIM-3 [100], in order to enhance clinical benefit. Moreover, efforts to suspend the non-T cell-associated TME-abundant inhibitory molecules, among others TGF-β, phosphoinositide 3-kinase gamma (PI3Kγ) and indoleamine 2,3-dioxygenase (IDO), have been found both safe and successful in combination with CIs [100-102]. Examined combinations of immune inhibitory and co-stimulatory strategies, as in the example of agonistically targeting OX-40 (CD134) or 4-1BB (CD137) [100, 103, 104], have shown promising results in the fight to improve the efficacy of CIs. Co-stimulatory molecules, as the aforementioned, are highly expressed in activated effector NK cells and CTLs and are expected to trigger proliferation and survival programs that battle CI treatment resistance.

Cell-based immunotherapy

Cell-based immunotherapies are today mostly T cell and DC oriented approaches, although NK cells have also been evaluated [105].

T cell immunotherapy is based on either isolating and expanding tumor-infiltrating lymphocytes (TILs) or directly modifying isolated naive T cells with TAA-specific TCRs or chimeric antigen receptors (CARs) [106]. The manipulated T cells are then returned to the patient where they are expected to act restrictively on the malignancy. Adoptive TIL transfer has been successfully used in clinical trials for the treatment of refractory metastatic melanoma showing durable complete regression for a fraction of patients [107]. However, *in vitro* expansion of bulk TILs entails the possibility to administer TILs with lower frequencies of tumor-reactive clones and an exhausted proliferation capacity [108]. Whole exome and RNA sequencing strategies aim to identify particular mutant-reactive clones in order to avoid the use of bulk TILs [108, 109]. Identification and cloning of such neoantigen-reactive TCRs opens the road for the creation of high-affinity engineered T cell armies directly from peripheral blood lymphocytes. It is highly possible that the immunosuppressive TME will pose a roadblock to TIL and TCR-transduced T-cell homing and cytotoxicity, thus combination of adoptive T-cell therapy and CIs will be an interesting field to explore [109].

Loss of MHC class I expression can also impede adoptive T-cell therapy and is often observed in therapy-refractory or progressing tumors. When a

poor MHC class I expression is limiting antigen-specific CTL responses, the exquisite recognition capacity of an antibody can be utilized via modifications of T cells with CARs [110]. CARs employ the antigen-recognition function of a single chain variable fragment (scFv) of an antibody, as substitute for the TCR extracellular domain, fused to classical intracellular domains for TCR-signaling and the CD28 and/or 4-1BB co-stimulatory molecules. CAR T cells thus bypass the need of MHC-dependent T-cell activation, expanding CTL recognizing abilities to theoretically any surface-expressed tumor-associated molecule. CAR T cells targeting CD19 show profound benefits for patients with hematological malignancies [111, 112]. Currently there are two FDA and EMA approved CAR T cell therapies, tisagenlecleucel (Kymriah®) for the treatment of pediatric and young adult patients up to 25 years of age with B-cell acute lymphoblastic leukemia (ALL) and patients with diffuse large B-cell lymphoma (DLBCL) and axicabtagene ciloleucel (Yescarta®) for the treatment of adult patients with relapsed or refractory DLBCL and primary mediastinal large B-cell lymphoma (PMBCL). Since CD19 is ubiquitously expressed on B cells, CAR T cells can ablate these cells in patients resulting in B cell aplasia. Though in this case this so called "on target/off tumor" effect is manageable, it is a major safety concern when it comes to CARs for solid malignancies. As TAAs are usually shared with normal tissues, CARs for solid tumors have been less successful, with additional homing and functionality issues posing extra challenges [113]. A major CAR T cell-associated adverse effect, associated also to neurotoxicity, is the cytokine release syndrome (CRS), which is practically the massive release of inflammatory cytokines, particularly IL-6, due to CAR T cells activation target elimination [113, 114].

On the antipode of adoptive transfer of TILs and gene-modified T cells is the DC approach. Herein antigen-specific T cells occur as a result of the vaccination with *ex vivo* modified antigen-loaded DCs or with agents for *in vivo* targeting of DC subsets [115]. As the present work is focusing on this approach, the next section "Dendritic cell vaccines" is dedicated on analyzing classical and novel DC vaccination strategies.

Dendritic cell vaccines

Main approaches

Two main efforts have been employed as DC vaccination in the context of cancer, one aiming to generate autologous antigen-loaded DCs *ex vivo* and vaccinate patients with them and a second aiming to deliver antigens and activate DCs *in vivo*.

DC cell-based vaccines

Ex vivo loaded DCs have been extensively tested in pre-clinical and clinical settings, allowing us to understand the impact of variations in vaccine preparation, such as choice of DC subset, antigenic cargo, maturation stimuli and route of administration, on therapeutic outcomes [115, 116]. Usually, monocytes or CD34$^+$ progenitors are used in order to generate *ex vivo* DCs for vaccine preparations. However, the transcriptional profiles of such DCs have revealed that vary significantly from the subsets of DCs found *in vivo*, suggesting caution in the selection of the proper DC subtype for the generation of vaccines [115, 117]. Loading of DCs *ex vivo* is usually accomplished by culturing immature DCs with tumor cell lysates and/or tumor-associated peptides, fusing DCs with incapacitated tumor cells or modifying DCs with vectors or mRNA sequences encoding TAAs or neoantigens. Loading with peptides directly on the MHC molecule requires knowledge of patient's HLA haplotype beforehand as there are defined epitopes binding to certain haplotypes. On the contrary, loading with whole tumor lysates or DC-tumor fusion requires the processing of antigens into multiple epitopes, which can benefit a multitude of HLA haplotypes. Maturation has been observed to be of immunological relevance to the development of successful *ex vivo* generated DCs, as observed for prostate and renal cancers [118]. Early vaccine preparations established a standard activation protocol including TNF-α, IL-1β, IL-6 and prostaglandin 2 (PGE2) [40]. As PGE2 was later found to promote Th2 polarization [119, 120] alternative maturation protocols emerged, utilizing combinations of cytokines with TLR stimuli, aiming for higher secretion of the Th1 polarizing cytokine IL-12 and induction of efficient CTL activation [38, 121, 122]. Comparisons between different administration routes have also provided important information on the optimal injection site and have highlighted the possibility that bystander DCs have a crucial role during DC vaccination [115, 123, 124]. Although *ex vivo* generated DC vaccines have been associated with suboptimal clinical responses despite inducing anti-tumor CTLs, FDA in 2010 and EMA in 2014 approved Sipuleucel-T (Provenge®), the first DC vaccine for metastatic castrate-resistant prostate cancer [125]. There are still certain challenges with this approach, such as a cumbersome and cost-defective production procedure, inefficient migration and/or exhaustion state by the time they meet T cells and immunogenicity of the loaded antigen, which may affect the quality of the induced T-cell responses [126, 127]. In the last years, methods to identify HLA-binding neoantigens inspired investigations in creating DC vaccines tailored for patient-specific mutations [128, 129]. In the era of CIs, neoantigen-vaccination with *ex vivo* generated DCs, along with regimens to alleviate the TME-induced immune suppression, may have the potential to be more effective than each therapy alone [130-132].

In vivo DC-targeting and non-targeted vaccines

In vivo, DCs can be selectively targeted and loaded with antigens coupled to antibodies for DC-specific surface receptors. Conjugation of TAAs with antibodies against for example lectin receptors such as the DC-specific ICAM-grabbing non-integrin (DC-SIGN) [133], the DEC-205 [134] and the CLEC9A [135] demonstrates the induction of both humoral and Th/CTL responses. As in the case of vaccination with DCs loaded *ex vivo*, maturation state of the targeted DCs has a profound impact on the generated immunity as absence of co-administered adjuvants may induce antigen-specific tolerance [136]. The addition of a maturation stimulus in the form of TLRs agonists or CD40 agonistic antibodies can nevertheless skew tolerance into robust anti-tumor immunity [135, 137]. Even in the absence of targeting DC-specific receptors, TLR-ligands aiming to activate DCs *in vivo* are often used as adjuvants for vaccines composed of neoantigens [138-140]. Agonists for CD40 or TLRs, such as polyI:C (TLR3), but also STING agonists, lipoplexes and pro-inflammatory cytokines, such as Flt3-L and GM-CSF, are some of the agents tested in order to activate DCs *in vivo* to effectively uptake tumor antigens and induce anti-tumor immunity [141-144]. Instead of vaccination with peptides and adjuvants, vaccine preparations such as GVAX and CRS-207 provide this combo of signals and have been found able to induce antigen-specific CTLs in pancreatic ductal adenocarcinoma patients [145] and other solid tumors. GVAX is composed of two irradiated pancreatic adenocarcinoma cell lines transduced to secrete GM-CSF, providing simultaneously a broad array of TAAs and a potent immune stimulating factor. The *listeria monocytogenes(Lm)*-based CRS-207 vaccine, can infect DCs and provide simultaneously antigenic-delivery and PAMP signals for the activation of DCs. Along the same line, oncolytic viruses harness the release of PAMP and DAMP signals when tumor cells are killed, which in combination with the expression of GM-CSF for the efficient maturation of DCs results in durable and objective responses in advance melanoma patients [146, 147]. Based on this outcome talimogene laherparepvec (T-VEC, Imlygic™), a genetically modified herpes virus that can replicate in melanoma cells and release GM-CSF, is the first oncolytic virus to gain approval from FDA and EMA.

Allogeneic DC vaccination

Results from clinical trials so far clearly indicate that DC vaccination, whether *in situ*- or with *ex vivo*-generated DCs, is safe and has the ability to generate anti-tumor immune responses but faces limitations in inducing satisfying clinical responses. As we begin to understand how the TME exerts immuno-suppressive effects we can speculate now that the limited clinical efficacy was most probably due to the fact that in most trials suboptimal

activated and antigen-loaded DCs were evaluated as monotherapies in advance stage patients. Successful DC vaccination relies on strong CTL induction, activation of Th cells and subsequent polarization to Th1, inhibition of T_{regs} and overcoming the immunosuppressive TME [148]. Interestingly, patient's endogenous bystander DCs seem to be central to the vaccine concept based on *ex vivo* loaded DCs, as endogenous DCs have been found to be the ultimate inducers of immunity [149-151], although in cancer patients immunosuppression is affecting their normal function [152].

By injecting allogeneic cells, or more specifically allogeneic DCs (alloDCs), one can awake a broad range of CTL and Th responses that create a strong pro-inflammatory environment inducing recruitment, as well as maturation, of bystander host DCs [153-155]. Around 7% of the total T cell repertoire is able to directly recognize and respond to allogeneic (from a different individual and therefore genetically unlike) MHC molecules expressed on the allogeneic cells [156]. This vigorous response, which appears to violate the rule of self-MHC restriction, is driven primarily by antigenic mimicry and engages both $CD4^+$ and $CD8^+$ T cells [157]. Notably, the inflammatory mediators induced by this allorecognition pathway, including TNF-α and IL-1β, have been shown to induce maturation of bystander immature monocyte-derived DCs *in vitro* [155]. Furthermore, the uptake and processing of allogeneic MHC-molecules by recipient's bystander DCs has been found to contribute to the indirect mode of allorecognition which is the dominant allogeneic response in patients with chronic transplant rejection [157].

Initially, allogeneic cell vaccination was proposed to take advantage of the surprisingly high precursor frequency of circulating alloreactive T cells [156] in order to induce tumor-specific responses [158]. It is speculated that since T cells with specificity against environmental non self-peptides exist among the alloreactive T cell population, it is rather possible that tumor-specific T cells may also become activated in cancer patients [158]. Moreover, the Th1 polarization and the general immunostimulatory environment created through the alloreaction can further support the activation of the cross-reacting tumor-specific CTLs and possibly overturn an immunosuppressive environment [155, 158]. One preliminary study in patients with advanced prostate cancer treated with skin allografts demonstrated this role of the allogeneic response [159]. From this study a possible mode of action is suggested, pointing that graft alloDCs can activate anergic neoantigen-specific T cells by chance via cross-reactivity, the phenomenon of one TCR responding vigorously to two or more peptide/MHC complexes, and the immunostimulatory microenvironment created from the allograft rejection. However, the cross-reactivity approach holds promise only for T cells activated by direct allorecognition of alloDCs in the skin allograft that bear no antigenic relationship with patient's cancer. In order to add a layer of antigenic-direction alloDCs can, for example, be fused with tumor cells as this way we can employ the potent immune enhancing abilities of the allorecog-

nition and the CD8^{+} T-cell activation due to MHC class I molecules of the autologous tumor cells [160, 161].

Interestingly, intratumoral vaccination with alloDCs without an antigenic load, utilizing the alloDCs as a pure adjuvant and the injected tumor as the source of tumor antigens, or vaccination with alloDCs pre-loaded with tumor antigens, with or without co-treatment with other immunotherapies has not been extensively studied. Clinical trials with partially matched allogeneic DCs, aimed to act as antigen-presenting cells, for patients with colorectal cancer [162] and B-precursor acute lymphoblastic leukemia [163] or fully allogeneic DCs for patients with metastatic renal cell carcinoma [164, 165], B-cell chronic lymphocytic leukemia [166], acute myeloid leukemia [167] and advanced hepatocellular carcinoma [168] have evaluated immunotherapy with alloDCs. Results indicate mixed responses, with most of the patients achieving stable disease accompanied by antigen-specific T cell responses.

In the majority of the aforementioned trials patients received intra-dermal, intra-nodal or intra-venous injections of alloDCs that already carried TAAs. In two clinical trials [165, 168] alloDCs were used simply as intra-tumoral injected adjuvants [169] and were evaluated as safe in terms of toxicity and capable to induce anti-tumor immunological responses. This approach aims on taking advantage of 1) the tumor site as a source of neoantigens [170] and 2) the need for strong bystander DC-activation in order to have effective responses [150, 151]. Intratumoral administration of immune primers can utilize the complete neoantigenic repertoire of a patient's tumor, thus minimizing the time-consuming step of characterization and *ex vivo* synthesis [170]. The ability of vaccine DCs to directly or indirectly interact with endogenous DCs is dependent on secretion of NK cell- and DC-recruiting inflammatory mediators [150]. Similar inflammatory mediators are also secreted by directly infected DCs in order to recruit and activate cross-priming non-infected bystander DCs during viral infections [171-173]. *In vitro*, such pro-inflammatory cytokines and chemokines can be secreted when monocyte-derived DCs are treated with different combinations of TLR ligands and IFN-γ [39, 165, 174, 175]. Thus, it is interesting to speculate that alloDCs activated in the presence of similar stimuli and used as an intratumoral adjuvant can promote bystander DC activation and anti-tumor immunity via the secretion of pro-inflammatory cytokines, further assisted by the Th1 licensing ability of the alloreaction [155]. The possibility to use a logistically effective approach, such as an off-the-self alloDC vaccine, will simplify the DC-vaccination process and shorten the time between diagnosis and treatment. The use of pro-inflammatory alloDCs as adjuvants for cancer immunotherapy, either alone or in combination with immunomodulatory antibodies, or along with an infection-enhanced adenovirus as a delivery vehicle for antigens, is the main focus of this work.

Method

Classical DC vaccination is based on the ability of autologous DCs to prime $CD8^+$ T cells via direct presentation and co-stimulation (Figure 4).
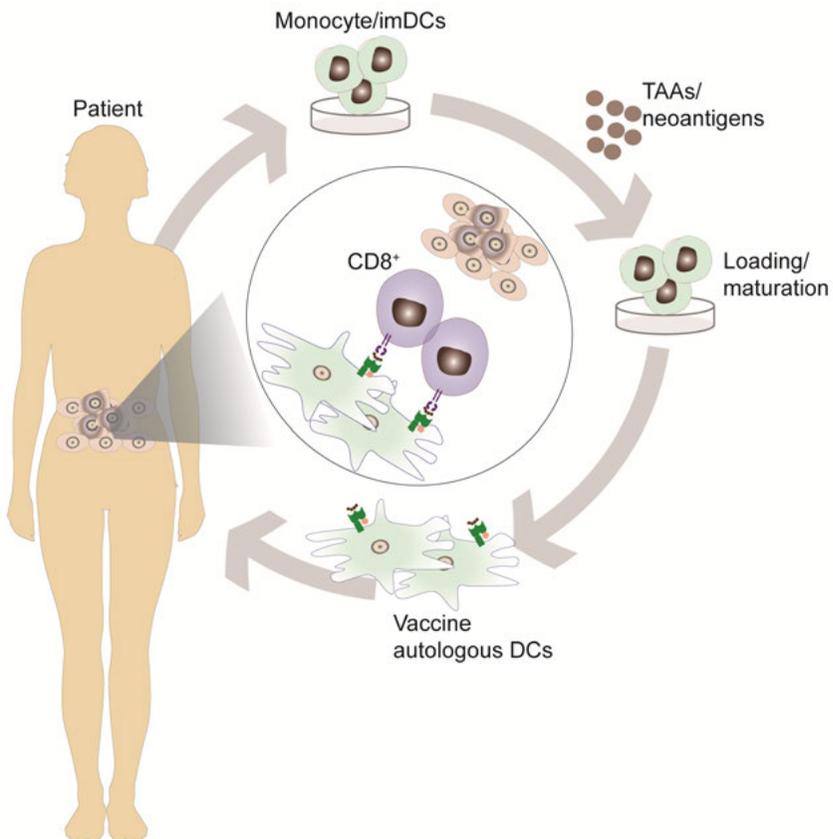
Figure 4. **Autologous DC vaccination.** In autologous DC vaccination, monocytes or immature DCs (imDCs) are isolated from the patient and after an *ex vivo* loading and maturation step they are injected back into the patient. According to this approach injected DCs are expected to directly present the loaded tumor-associated antigens (TAAs) or neoantigens to antigen-specific T cells, thereby eliciting a tumor-specific T cell response.

However, there are indications suggesting that transferred vaccine DCs are very poor in priming cytotoxic responses themselves, but instead they act as immunogenic primers affecting recruited endogenous bystander DCs [150, 151]. This priming effect of vaccine-DCs on bystander DCs is mostly based on secreted pro-inflammatory stimulators [150] and may occur independently of MHC-compatibility [173]. Thus, we attempted to evaluate the immune-priming properties of allogeneic DCs (alloDCs) (Figure 5) that have been matured in the presence of a Th1-type polarizing cocktail of TLR ligands and IFN-γ [39, 174]. An infection-enhanced serotype-5 adenovirus vector with fiber shaft and knob from serotype-35 and a hexon (major capsid protein) modification to enhance transduction efficacy [176] was used to evaluate the feasibility to load alloDCs in order to provide simultaneously TAA sequences.
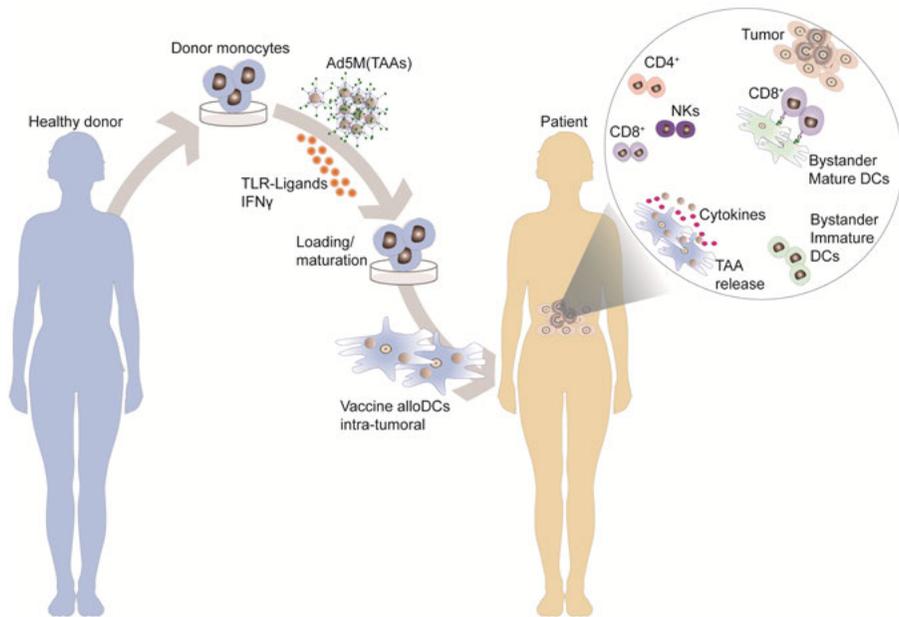
Figure 5. **Vaccination with allogeneic DCs.** Vaccination with pro-inflammatory allogeneic DCs (alloDCs) starts with the isolation of monocytes from a healthy allogeneic (unrelated to the patient) donor. The pro-inflammatory state of alloDCs is induced by a combination of Toll-like receptor (TLR) ligands and IFN-γ, while loading of alloDCs with tumor antigens is facilitated by an infection-enhanced adenovirus (Ad5M). When alloDCs are injected subcutaneously or intratumorally they are expected to recruit and activate immune cells, including NK cells, T cells and bystander DCs. AlloDCs will be destroyed as they are allogeneic. If the alloDCs are pre-loaded with tumor antigens, these will be released into the micro-environment, along with tumor-antigens released from the tumors (if injected intratumorally due to killing mediated by activated NK cells). Recruited bystander DCs will become activated in this pro-inflammatory environment and can capture antigenic-material and subsequently cross-prime tumor-specific T cells.

We speculate (Figure 6) that in cancer patients, intratumorally injected alloDCs will directly activate patient's recruited immune cells, including NK cells and alloreactive T cells, within the injection site due to their active secretion of pro-inflammatory cytokines and chemokines. NK cells activated by the alloDCs may be induced to locally destroy tumor cells (and also the alloDCs), thus promoting the release of tumor antigens. The cross-talk between alloDCs and recruited NK cells and alloreactive T cells will lead to additional production of pro-inflammatory factors that may recruit and activate endogenous DCs, promote cross-presentation of the acquired tumor antigens and possibly counteract immunosuppressive components of the local TME.
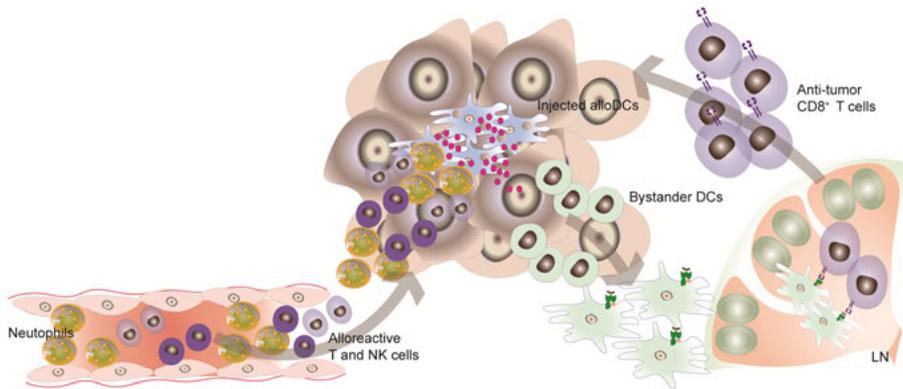
Figure 6. **Proposed mode of action for intratumoral alloDCs.** After administration intratumorally, alloDCs will secrete Th1-associated chemokines and cytokines that recruit immune cells such as NK cells, T cells, neutrophils and DCs. The interaction between recruited NK cells and alloDCs will induce NK cell-mediated killing of the alloDCs, as well as killing of the adjacent tumor cells. Tumor-antigens released in the tumor environment, along with the released Th1 cytokines, can induce maturation and improve cross-presentation of recruited bystander endogenous DCs. Such DCs can lead to the priming of tumor-specific cytotoxic CD8$^+$ T cells.

Summary of investigations

Paper I

Aim: Recent experimental and clinical results support an important role for endogenous bystander dendritic cells (DCs) in the efficiency of autologous patient-derived DC-vaccines, as bystander DCs take up material from vaccine-DCs, migrate to draining lymph node and initiate antitumor T-cell responses. In Paper I we aimed to evaluate the possibility of using allogeneic DCs as vaccine-DCs to activate bystander immune cells and promote antigen-specific T-cell responses.

Main findings: We found that human DCs matured with a cocktail of the toll-like receptor ligands, polyI:C and R848, and IFN-γ (COMBIG) alone or in combination with an infection-enhanced adenovirus vector (Ad5M) exhibit a pro-inflammatory state. COMBIG and COMBIG/Ad5M-matured allogeneic DCs (alloDCs) efficiently activated T cells and NK cells in allogeneic co-culture experiments. The secretion of immunostimulatory factors during the co-culture promoted the maturation of bystander-DCs, which efficiently cross-presented a model-antigen to activate antigen-specific CD8$^+$ T cells *in vitro*. The most important finding of Paper I is that alloDCs, alone or in combination with Ad5M as loading vehicle, may be a cost-effective and logistically simplified DC-vaccination strategy to induce anti-tumor immune responses in cancer patients.

Paper II

Aim: Autologous patient-derived dendritic cells (DCs) modified *ex vivo* to present tumor-associated antigens (TAAs) are frequently used as cancer vaccines. However, apart from the stringent logistics in producing DCs on a patient basis, accumulating evidence indicate that *ex vivo* engineered DCs are poor in migration and in fact do not directly present TAA epitopes to naïve T cells *in vivo*. Instead, it is proposed that host bystander-DCs take up material from vaccine-DCs, migrate and subsequently initiate antitumor T-cell responses. In Paper II we aimed to use mouse models to examine the effect of alloDCs in host DC-activation and promotion of antigen-specific T-cell immunity *in vivo*.

Major findings: We found that alloDCs were able to initiate host bystander-DC activation and migration to draining lymph nodes leading to T-cell activation. The pro-inflammatory milieu created by alloDCs also led to recruitment of NK cells and neutrophils at the site of injection. Vaccination with alloDCs combined with Ad5M(gp100), an infection-enhanced adenovirus encoding the human melanoma-associated antigen gp100 resulted in generation of CD8$^+$ T cells with a T-cell receptor specific for the gp100$_{25-33}$ epitope. Ad5M(gp100)-alloDC vaccination in combination with transfer of gp100-specific pmel-1 T cells resulted in prolonged survival of melanoma-bearing mice and altered the composition of the tumor microenvironment (TME). The most important finding of Paper II is that alloDCs together with TAA- or neoepitope-encoding Ad5M can become an "off-the-shelf" cancer vaccine, which can reverse the TME-induced immunosuppression and induce host cellular anti-tumor immune responses in patients without the need of a time-consuming preparation step of autologous DCs.

Paper III

Aim: The induction of anti-tumor immune responses, especially those mediated by effector tumoricidal T cells, is considered necessary for effective immunotherapies. However, cytotoxic T cells alone cannot function properly in the presence of an immunosuppressive tumor microenvironment (TME). Human Papillomavirus (HPV)-associated tumors embody this problem as the presence of the viral E6 and E7 antigens renders HPV-associated tumors immunogenic and induces anti-tumor T cells, however with a limited effectivity due to hindrance from the immunosuppressive TME. In Paper III we aimed to examine the prophylactic and therapeutic potential of alloDCs as immune primers and an adenoviral vector encoding for HPV-specific sequences (Ad5(E6/7)).

Main findings: We found that the co-administered immunotherapy could efficiently induce HPV-specific T cells, which led to complete tumor protection in a prophylactic setting and increased tumor regression in a therapeutic setting. In addition, the co-administration therapy can alter the TME in favor of anti-tumor immunity. TME alteration included the influx of CD8$^+$ T cells, the majority of which exhibited specificity for a viral E7 epitope recognized from murine T cells, and the cross-presenting CD103$^+$ subset of DCs. Most importantly, in Paper III we found that NK cells may be crucial in the mechanism through which alloDCs recruit effector bystander immune cells in the TME.

Paper IV

Aim: Cancer vaccination and treatment with immuno-modulating antibodies are two promising immunotherapies, which can have synergistic effects in combination strategies. In Paper IV we aimed to evaluate whether intra-tumorally injected allogeneic dendritic cells (alloDCs) can be used as an immune primer in combination with anti-PD1 immune checkpoint antibodies or anti-CD137 co-stimulation antibodies, to enhance the therapeutic responses in a murine colon cancer model.

Major findings: We found that combined therapy resulted in delayed tumor progression and prolonged survival in the case of anti-PD1 and the complete regression of CT-26 tumors in 3 out of 9 mice in the case of anti-CD137. Moreover, mice exhibiting complete responses were subsequently protected from tumor re-challenge. Notably, in Paper IV we found that increased expression of CD137 on human NK cells and T cells after co-culture with human alloDCs suggests that these synergistic effects observed in the murine setting could be translated into an effective therapeutic strategy in cancer patients.

Future perspectives

Allogeneic DCs combined with CIs or co-stimulatory molecules

Tumors can be separated according to immune cell-infiltration, and particularly T-cell infiltration, into immune-inflamed, -excluded and -deserted. These infiltration-patterns can affect the outcomes of immunotherapeutic approaches [78]. Checkpoint and co-stimulation immunotherapies can be very effective in tumors that are immune-inflamed, as exhausted CTLs and tumor cells express the necessary molecules for the blockade to be effective (for example PD-1 on infiltrated T cells/PD-L1 on tumor cells). However, immune-excluded and -deserted tumors need an additional pre-treatment in order to break the exclusion and bring immune infiltrates into the tumor core. We are interested in testing the local and systemic effect of alloDCs, alone or in combination with other immunotherapies, in tumors with different types of microenvironments. We aim to evaluate in more depth how alloDCs are influencing effector functions of exhausted T cells and NK cells, simulating a more realistic scenario as for the case of immune-inflamed tumors. Checkpoint inhibitors, targeting PD-1 for example, or antibodies targeting co-stimulatory molecules, such as 4-1BB, and/or myeloid depletion regimens administered simultaneously or sequentially might help us evaluate the optimal timing of priming the TME and effector functions of CTLs in order to obtain the most in terms of therapeutic outcomes.

Allogeneic DCs and adoptive T-cell transfer

Adoptive transfer of CAR cells has seen tremendous advances in the last years for B-cell malignancies. However, their efficacy for solid tumors is severely hampered by homing-roadblocks posed by the TME. There are indications that an increase of chemokines and cytokines in the tumor might affect the recruitment of adoptively transferred T cells to the tumor site [177]. We are interested to evaluate deeper how alloDC administration is changing the cytokine/chemokine milieu of the TME and the function and status of the tumor vasculature, as well as how these affect adoptive transfer of CAR, or TCR engineered, T cells.

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