Activating the Sting Pathway

a potential strategy for anti-cancer immunotherapy?

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A POTENTIAL STRATEGY FOR ANTI-CANCER IMMUNOTHERAPY?

BY
MARLENE FYRSTENBERG LAURSEN

DISSERTATION SUBMITTED 2020

AALBORG UNIVERSITY
DENMARK

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A POTENTIAL STRATEGY FOR ANTI-CANCER IMMUNOTHERAPY?

by

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M. Fyrstenberg Laursen, E. Kofod-Olsen and R. Agger. Activation of dendritic cells by targeted DNA: a potential addition to the armamentarium for anti-cancer immunotherapy. Cancer Immunology, Immunotherapy, vol. 68 (11), pp. 1875-1880, 2019

CONFERENCE ABSTRACTS

Laursen, Marlene Fyrstenberg; Agger, Ralf; Kofod-Olsen, Emil. Investigations on a Novel Dendritic Cell-Targeted Adjuvant for Anti-Cancer Therapy. Conference 18th Progress in Vaccination Against Cancer (PIVAC-2018), October 2018, Oslo, Norge

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Laursen, M. F., Kofod-Olsen, E. & Agger, R. The delivery of double-stranded DNA to dendritic cells and its effect on dendritic cell maturation. Danish Society of Immunology, Annual Meeting, April 2016, Copenhagen, Denmark. Danish Society of Immunology, s. 21 No. 18

Fredriksen, L., **Laursen, M. F.**, Birkelund, S., Agger, R. & Kofod-Olsen, E. CD11c-targeted dendritic cell vaccine. Danish Society of Immunology, Annual Meeting, May 2019, Copenhagen, Denmark.

Zeiler, C., Banasik, A., **Laursen, M. F.**, Agger, R. & Kofod-Olsen, E. Analysis of Type 1 IFN Production by DCs after Stimulation with Supernatant of Necroptotic Cells. Danish Society of Immunology, Annual Meeting, May 2019, Copenhagen, Denmark.

Nielsen, L., **Laursen, M. F.**, Agger, R. & Kofod-Olsen, E. Generation of a recombinant CD11c-targeted vaccine containing SIINFEKL antigen. Danish Society of Immunology, Annual Meeting, May 2019, Copenhagen, Denmark.

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ENGLISH SUMMARY

Immunity and cancer are known to be linked together in a close relationship. The knowledge of how the immune system interacts in tumor development and elimination has led to a revolution in cancer treatment with development of anti-cancer immunotherapy. The potential of immunotherapy against cancer has been confirmed by checkpoint blockade therapy, which can increase survival of cancer patients, by lifting the suppression of T cell responses that is generated by the cancer cells. However, some patients still fall short in responding to the treatment, and development of new approaches to supplement the function of checkpoint blockade is of high interest. Dendritic cells (DCs) are highly involved in immune regulation of cancer, specifically since they are professional antigen-presenting cells and T cell activators and able to shape the character of the immune response. During anti-cancer immune responses, type I interferons (IFN-I) have been shown to be important in the activation of DCs and to increase their ability to mediate T cell dependent tumor rejection. The molecule Stimulator of Interferon Genes (STING) is a major facilitator of IFN-I production in many different cell types, including DCs. Targeting DCs and STING to increase IFN-I production and immune activation could therefore potentially be a way to enhance anti-cancer immune reactions.

The possibility of utilizing DCs and STING in immunotherapeutic cancer treatment was investigated through two studies. In the first study, an adjuvant construct targeted to DCs was generated. The construct consisted of an antibody specific for DC surface molecule CD11c which was conjugated with dsDNA, a known activator of STING, and the maturation status of the cells was determined. Here it was seen that targeting STING with dsDNA in DCs led to a STING-dependent increase in maturation and enhanced their ability to stimulate T cells. In the second study, direct STING agonist, cyclic GMP-AMP (cGAMP), were used together with different cholesterol inhibitors to activate DCs and macrophages. It was found that cholesterol inhibitor treatment led to an increased STING sensitivity to cGAMP treatment, due to increased ER sequestering of STING. The combination treatment with cGAMP and cholesterol inhibitors strongly increased the maturation of human and murine DCs and the activation of human macrophages *in vitro*. When cholesterol inhibition therapy was applied *in vivo* for treatment in a murine tumor model, a reduction in tumor growth was detected.

Collectively, these studies indicate that activation of DCs in a STING dependent manner is possible by targeting dsDNA directly to the cells, or by exposing the cells to cGAMP. Furthermore, in the latter situation, the maturational response can be enhanced by the addition of cholesterol inhibitors. Possibly, these methods of increasing the T cell-activating capacity of DCs could be a valuable addition to immunotherapeutic treatment against cancer.

DANSK RESUME

Det er kendt, at immunitet og kræft er forbundet i et tæt forhold. Viden om hvordan immunsystemet er involveret i udviklingen og elimineringen af tumorer har ført til en revolution inden for kræftbehandling med udviklingen af anti-kræftimmunterapi. Potentialet i immunterapi mod kræft er blevet velbekræftet via checkpoint blokade, som kan føre til øget overlevelse af kræftpatienter ved at løfte kræftcellernes undertrykkelse af T celler. Dog forholder det sig således, at hos nogle patienter ses der kun ringe effekt af denne behandlingstype, og det er derfor af høj interesse at finde nye tiltag og udvikle ny terapi, der kan bruges som supplement til checkpoint blokade-terapien. Dendritceller (DC) er stærkt involveret i den immunologiske regulering af kræft, specielt eftersom de er professionelle antigen-præsenterende celler der kan aktivere T celler og hermed kan forme immunresponset. Type I interferoner har vist sig at være meget vigtige i aktiveringen af DC'er og kan øge deres evne til at formidle den T celle afhængige eliminering af tumorer. Molekylet Stimulator of Interferon Genes (STING) er en vigtig facilitator af type I interferon-produktionen i mange forskellige celletyper, inklusiv DC'er. Målretning af terapi mod DC'er og STING med henblik på at øge type I interferonproduktionen og aktiveringen af immunresponser kan derfor meget vel være en god behandlingsmåde til at styrke den immunologiske krig mod kræft.

Gennem to studier har vi undersøgt muligheden for at målrette terapi mod DC'er og STING som et muligt bidrag til den immunterapeutiske kræftbehandling. I det første studie konturerede vi et DC-målrettet adjuvans. Det konstruerede adjuvans bestod af et antistof specifikt rettet mod et DC-overflademolekyle, CD11c, som blev konjugeret med dsDNA, der er kendt for at kunne aktivere STING, og vi analyserede modningsstatus af DC'erne. Vi så, at målretning af dsDNA mod DC'er førte til øget modning af cellerne, afhængig af STING, og ydermere øgede det cellernes evne til at stimulere T celler. I det andet studie anvendte vi den direkte STING agonist, cGAMP, i kombination med kolesterolhæmmer, til at aktivere DC'er og makrofager. Vi så, at behandling med kolesterolhæmmer førte til en øget sensitivitet af STING for cGAMP behandling, via en øget frigivelse af STING fra ER. Kombinationsbehandlingen havde en stærkt forøgende virkning på modningen af humane og murine DC'er og aktiveringen af humane makrofager *in vitro*. Når kolesterolhæmmer terapi blev anvendt *in vivo* til behandling i en murin tumor model observerede vi en reduktion i tumorvækst.

Samlet set peger disse studier mod, at en STING-afhængig aktivering af DC'er er mulig ved målretning af DNA mod cellerne via CD11c og ved at anvende kombinationsbehandling med kolesterolhæmmer og cGAMP. Begge behandlingsmetoder kan være værdifulde tilføjelser til det immunterapeutiske forsvar mod kræft.

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Review I: **M. Fyrstenberg Laursen**, E. Kofod-Olsen and R. Agger, "Activation of dendritic cells by targeted DNA: a potential addition to the armamentarium for anti-cancer immunotherapy", *Cancer Immunology, Immunotherapy, pp. 1-6, 2019*

Manuscript II: **M.F. Laursen*,** E. Nilsson*, B. Zhang, A. Etzerodt, A. Banasik, C. Zeiler, P. Owczarska, M. Jahn, R. Agger, S. R. Paludan, E. Kofod-Olsen and M. R. Jakobsen. "Inhibition of Membrane Cholesterol Primes STING by Endoplasmic Reticulum Sequestration and Inhibits Growth of Murine Subcutaneous Tumors" *[Manuscript in preparation]*

*Shared Authorship

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
BMDC	Bone Marrow-Derived Dendritic Cell
cDC	Conventional Dendritic Cell
CDN	Cyclic di-Nucleotide
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP Synthase
CLR	C-type lectin receptor
cMoP	Common Monocyte Progenitor
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
ER	Endoplasmic Reticulum
iDC	Immature Dendritic Cell
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
IRF	Interferon Regulatory Factor
LC	Langerhans Cell
mDC	Mature Dendritic Cell
MDP	Macrophage and Dendritic Cell Progenitor
MDSC	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
moDC	Monocyte-Derived Dendritic Cell
MPS	Mononuclear Phagocyte System
MβCD	Methyl-Beta Cyclodextrin
NK Cells	Natural Killer Cells
OVA	Ovalbumin

PAMP	Pathogen Associated Molecular Pattern
PD-1	Programmed Cell Death Protein
pDC	Plasmacytoid Dendritic Cell
PD-L1	Programmed Death Ligand 1
STING	Stimulator of Interferon Genes
TA	Tumor Antigen
TGF-β	Transforming Growth Factor Beta
TME	Tumor Microenvironment
Tregs	Regulatory T Cells
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1. INTRODUCTION

The immune system plays an important role in the development and elimination of cancer and it is quite evident that the immune system can be modulated with therapy, leading to enhanced immunological elimination of tumors. This became especially clear in 2018, when the Nobel Prize in Medicine or Physiology was awarded to James P. Allison and Tasuku Honjo for their development of checkpoint inhibition, a type of immunotherapy which is now used in the treatment of several cancer types [1]. To utilize the immune system for cancer treatment, it is important to understand the complexity of why people develop cancer and how the immune system is involved in this process.

The following introduction will try to elucidate the connection between immunity and cancer and take a closer look on how therapeutic approaches can increase activation of the immune system to fight cancer. The focus will be on how dendritic cells (DCs) can be activated and how this may improve immune responses against cancer. A review of the STING signaling pathway will be given with the goal of explaining how this pathway is involved in the linkage between innate and adaptive immunity. How therapy aiming to activate dendritic cells and the STING pathway can be an option for cancer treatment is what this thesis pursues to clarify.

1.1. IMMUNITY MEETS ONCOLOGY

Why do people develop cancer? This is a question that researchers have been seeking to answer for many years. Hanahan and Weinberg [2, 3] have summarized The Hallmarks of Cancer, which explains how it is possible for cancers to emerge. Originally, the theory described six hallmarks or alterations in the cell's physiology, which can cause a normal cell to develop into a cancer cell [2]. Several factors can induce this transformation, including viral infections, exposure to carcinogens or radiation, innate genetic defects or chronic inflammation [4]. After a decade of research, The Hallmarks of Cancer theory was expanded to include even more hallmarks. One of the new hallmarks described in the expanded theory is called Evasion of Immune Destruction [3]. This highlights that there is indeed a relationship between the immune system and cancer, and that the ability of tumor cells to avoid the immune system is a key factor involved in the establishment of clinically detectable malignancies.

1.1.1. CANCER IMMUNITY

The connection between the immune system and cancer has been recognized for several decades. Back in the 1950's, Burnet and Thomas [5, 6] started the groundwork which would eventually lead to the development of the concept of immunological

surveillance, which was summarized by Burnet [7] in 1970. Years of research on the link between immune- and tumor cells followed, and the immunological surveillance concept was updated in the beginning of 2000 by Dunn and colleagues [8], who proposed the new: Cancer Immunoediting Theory. This theory describes the consequences of the meeting between immunity and cancer as a three-phase process, referring to what happens to the tumor cells, which can either be eliminated, become part of an equilibrium or escape [4, 8]. The latest update of the theory about immuno-oncology was made by Chen and Mellman [9], who introduced the Cancer Immunity Cycle. This theory describes a 7-step process of how the immune system can effectively eliminate tumor cells. As such, this theory does not abolish the cancer immunoediting theory, but rather introduces a more detailed description of the elimination phase [9].

1.1.2. ELIMINATE, EQUILIBRATE, ESCAPE

Understanding the relationship between the immune system and tumor cells, especially in the elimination- and escape phase, is crucial to lay out the groundwork to develop effective immunotherapy (**Figure 1**).

Within the first phase of immunoediting, *elimination*, lies the original theory of immunosurveillance, and the cancer immunity cycle can be used to describe the phase in more detail. In the elimination phase, the immune system is alerted to the presence of tumor cells. Innate immune cells infiltrating the tumor site starts the process. Macrophages produce IL-12, which can activate Natural Killer Cells (NK cells) and Natural Killer T Cells (NKT cells), which play an important role at this stage. This has been revealed in studies where depletion of NKT and/or NK cells results in increased susceptibility to tumor development in a methylcholanthrene (MCA) induced tumor model [10, 11]. NK cells can induce perforin-dependent cytotoxicity, and in a perforin lacking mouse model, tumor growth is accelerated [12], confirming the important role of these innate immune cells. Similarly, in models lacking γδ T cells tumors are more prone to develop [13]. γδ T cells are a source of IFN-γ, a cytokine playing a central role in immunosurveillance [14]. Mice lacking the IFN-γ receptor subunit or the transcription factor Stat1, leading to disruption of the IFN-γ signaling pathway, develop tumors more rapidly than wild type mice [15]. Similarly, mice lacking the IFN-I receptor subunit, making them insensitive to IFN-I, are more susceptible to tumor development [16].

The generation of an inflammatory environment at the tumor site promotes cell death, resulting in the release of tumor antigens (TAs) and danger signals [17]. Once the tumor cells start releasing TAs, the initiation of an adaptive anti-tumor immune response commences. The cancer immunity cycle, introduced by Chen and Mellman [9], extends the description of the elimination phase through seven steps: In step **1**, TAs are released from the tumor cells, and together with danger signals, these are captured by DCs. In step **2**, activated DCs travel to lymph nodes and present TAs to

naïve T cells via MHC-I and II molecules. When T cells have recognized the presented TA, they are primed and activated in step **3**. Activated T cells travel via the blood stream to the tumor site in step **4** and infiltrate the tumor site in step **5**. In step **6** the T cells meet and recognize the tumor cells, which leads to step **7,** where the tumor cells are eliminated by the T cells [9]. The killing of tumor cells completes the cycle and causes the release of more TAs and danger signals, leading to epitope spreading, and thus the cycle starts over again [9]. Especially important in the killing of tumor cells are the T cells. The role of lymphocytes is clearly shown in models containing knockout of the RAG2 gene [18], resulting in a complete lack of functional lymphocytes. The lack of lymphocytes results in enhanced tumor growth and the generation of more immunogenic tumors, compared to immunocompetent controls [19]. Furthermore, it has been shown that cancer patients, which have high amounts of tumor-infiltrating T cells, have a better prognosis than patients with low T cell infiltration [20].

The elimination phase allows the host's immune system to eradicate tumor cells, however, if not successful, it will lead the immune system and the tumor cells into an *equilibrium*, forming more vicious non-immunogenic tumors. The pressure put on the tumor cells by T cells and IFN-γ is likely to kill all immunosensitive tumor cells, but spare mutated non-immunogenic tumor cells. The mutated tumor cells, resistant to immune destruction, can expand, and this leads to the last phase of immunoediting, *escape* [8].

The *escape* phase arises when tumor cells develop sneaky ways to hide pugholmlves from the immune system. Tumor cells secrete factors that have immune suppressive effects. These factors include Vascular Endothelial Growth Factor (VEGF), which weakens DC function [21], Indoleamine 2,3-Dioxygenase (IDO), hampering T cell proliferation through the metabolism of tryptophan [22], and MHC Class I Chain Related-Protein A/B (MICA/B), that binds to Natural Killer Group 2D Receptor (NKG2D), expressed on NK, γδ T and αβ T cells, suppressing their function [23]. Tumor cells also have the ability to directly avoid T cell cytotoxic elimination, via upregulation of Programmed Death Ligand 1 (PD-L1), which interacts with surface receptors on T cells, inducing apoptosis in the cells [24]. Furthermore, immune cells can become tolerogenic in the tumor microenvironment (TME). This can be caused by the presence of the tolerogenic cytokines, Transforming Growth Factor β (TGF-β) and Interleukin 10 (IL-10). TGF-β and IL-10 draw macrophages into the tumor site, which in turn increases the generation of TGF-β and IL-10. TGF-β induce suppression of NK cells, DCs and T cells. This cytokine also leads to the generation of Regulatory T Cells (Tregs), which further promotes the tolerogenic environment through the production of TGF-β and IL-10. Tregs also directly inhibit T cells via expression of PD-L1 and Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4), which promote T cell anergy [25]. Myeloid-Derived Suppressor Cells (MDSC) attracted to the tumor site by the immunosuppressive factors contribute to the production of TGF-β, leading to anergy of NK- and T cells and generation of Tregs [26, 27].

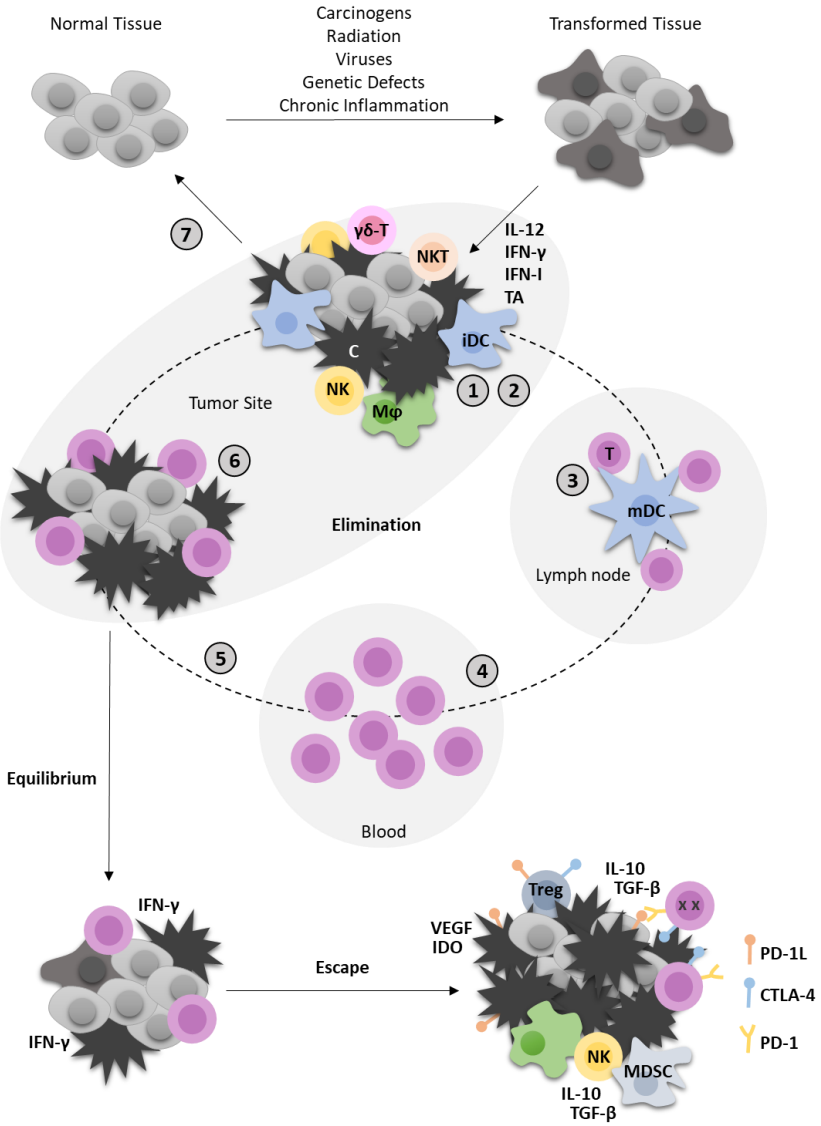

Figure 1. Overview of the interplay between tumor- and immune cells during immunoediting [8]. Tissue being transformed enter the *elimination* phase where the immune system can restore normality of the cells. This is described through the seven steps in the cancer immunity cycle [9]. **1.** DCs capture antigens and danger signals released at the tumor site. **2.** Activated DCs travel to lymph nodes and present tumor antigens to T cells. **3.** Leading to T cell priming and activation. **4.** Activated T cells proliferate and travel to the tumor site. **5.** T cells infiltrate the tumor site. **6.** T cells interact and recognize the tumor cells. **7.** T cells induce cytotoxic killing of the tumor cells. Transformed tissue can enter the *equilibrium* phase leading to generation of mutated non-immunogenic tumors and the entry into the *escape* phase where several suppressive mechanisms lead to the development of clinically apparent malignancies. Abbreviations: **γδ-T:** γδ-T cell. **NKT:** NKT cell. **NK:** NK cell. **iDC:** immature dendritic cell. **Mφ:** macrophages. **T:** T cell. **mDC:** mature dendritic cell. **Treg:** Regulatory T cell. **MDSC:** Myeloid-Derived Suppressor Cells.

The main goal of immunotherapy against cancer is to lift the immunosuppressive environment created at the tumor site, so the immune system can reactivate the cancer immunity cycle and eliminate the cancer. Novel therapies targeting different parts of the immune system, involved in immunoediting, have been developed, and most striking is perhaps the checkpoint blockade therapy, which is being used for treatment of a range of different cancer types. This therapy utilizes monoclonal antibodies targeted against CTLA-4, PD-1 or PD-L1, lifting the suppressive hold of T cells [28-30]. The use of checkpoint blockade in the clinic has proven to be useful. However, some patients still fall short in responding to the treatment and focusing on developing new therapies targeting other parts of the immune system, could be the next step in the immunological fight against cancer. A cell that plays a major role in the meeting between immunity and cancer is the DC since these cells can connect the innate- and adaptive immune system.

1.2. THE DENDRITIC CELL

Once upon a time in the 1970's, Ralph Steinman and Zanvil Cohn, made the discovery of a novel cell type in the spleen of mice. The characteristic morphology of this cell type, with its stellate appearance, made it stand out from the heterogenous group of spleen cells. Through *in vitro* and *in vivo* studies of the cells' morphology, it became clear that its distinct appearance was owed to its dynamic dendritic cell processes, giving rise to the name and the tale of this novel cell type: the Dendritic Cell (DC) [31].

1.2.1. ONTOGENY AND SUBTYPES

In the beginning of the 1970's, a classification system for macrophages and monocytes, named the Mononuclear Phagocyte System (MPS), saw the light of day [32]. With the discovery of DCs, these joined the MPS [33]. DCs are located in blood, lymphoid- and non-lymphoid tissue, and their primary role is to form a link between the innate- and adaptive immune system [34]. During the last five decades, intensive research has been carried out to elucidate the role and function of DCs. DCs are a very heterogeneous group of cells and the need for a common nomenclature and subdivision of DC types has been necessary. This, however, has proven to be a rather difficult assignment and considerable disagreement on how to subdivide DCs has also led to considerable confusion for many researchers [35]. The fact that a direct translation from the murine system to the human system does not seem possible, has further complicated the issue. However, in recent years, a broad consensus about ways to subdivide the heterogenous group of DCs based on ontogeny, phenotype, location and/or function, has emerged [35, 36].

As suggested by Guilliams et al. [35], a way to start the process of division, is by looking at the development of the cells within the MPS. During the last couple of decades, the ontogeny of murine DCs, monocytes and macrophages has been mapped,

and this provides researchers with a type of classification system of these cells. It has been suggested that the ontogeny of DCs is conserved across species [37], and therefore the classification based on ontogeny can be applied in both the human and the murine system.

DCs have a short lifespan and are therefore constantly being renewed. This process starts in the bone marrow where they develop from a distinct hematopoietic lineage [36]. The different stages of development are defined by the cell phenotype (**Figure 2**). In 2006 [38], it was discovered that cells within the MPS originate from one common progenitor termed the macrophage and dendritic cell progenitor (MDP). This cell type is defined as being negative for CD3, CD19, CD54 and CD14 lineage markers (Lin^-), and by the expression of CX_3CR1 and c-Kit (or CD117) [33, 38, 39]. Following the discovery of MDPs, another progenitor cell was found, which specifically gives rise to DCs, originally described with the term pro-DC [40], but later the name Common Dendritic Cell Progenitor (CDP) was adapted to the terminology. CDPs are defined as $Lin^-c\text{-}Kit^{int}$ and by the expression of M-CSFR (or CD115) and Flt3 (or CD135) [40, 41]. The terminology and subdivision of DCs applied here is based on Guilliams et al. [35] and includes: plasmacytoid DCs (pDCs) and conventional DCs (cDCs), which can be further divided into Conventional Type 1 DCs (cDC1) and Conventional Type 2 DCs (cDC2). A precursor cell for cDCs has been identified in the bone marrow, blood, spleen and lymph nodes [42]. This precursor cell gives rise only to cDCs and not pDCs. It is defined as $Lin^-SIRP\text{-}\alpha^{int}CD11c^+MHC\text{-}II^-Flt3^+$, and is termed preDC [42, 43].

In addition to pDCs and cDCs, another DC subtype derived from monocytes, termed monocyte-derived dendritic cells (moDCs), develops during inflammatory conditions [33]. Previously, it was thought that monocytes developed directly from MDPs. However, a new progenitor cell was identified in mouse bone marrow and spleen. This cell gives rise to monocytes, but not any of the DC subtypes. This progenitor cell, termed common monocyte progenitor (cMoP), is defined as $Lin^-cKit^+M\text{-}CSFR^+Flt3^-LyC6^+CD11b^-$, but also expresses CX_3CR1, and is negative for DC markers, including CD11c and MHC-II [44].

The four DC subtypes, namely the moDC, pDC, cDC1 and cDC2, have different phenotypes and functions, and their differentiation depends on different transcription factors and cytokines (**Figure 2**). Furthermore, when defining the different DCs, tissue-location is often an included factor. As mentioned, it is not doable to translate directly from mouse to human when defining DCs. Murine DCs are the most well studied and well defined in terms of phenotype and function. In the following, the division of DC subtypes, based on all these factors, distinguishing between the human and murine system, will be given.

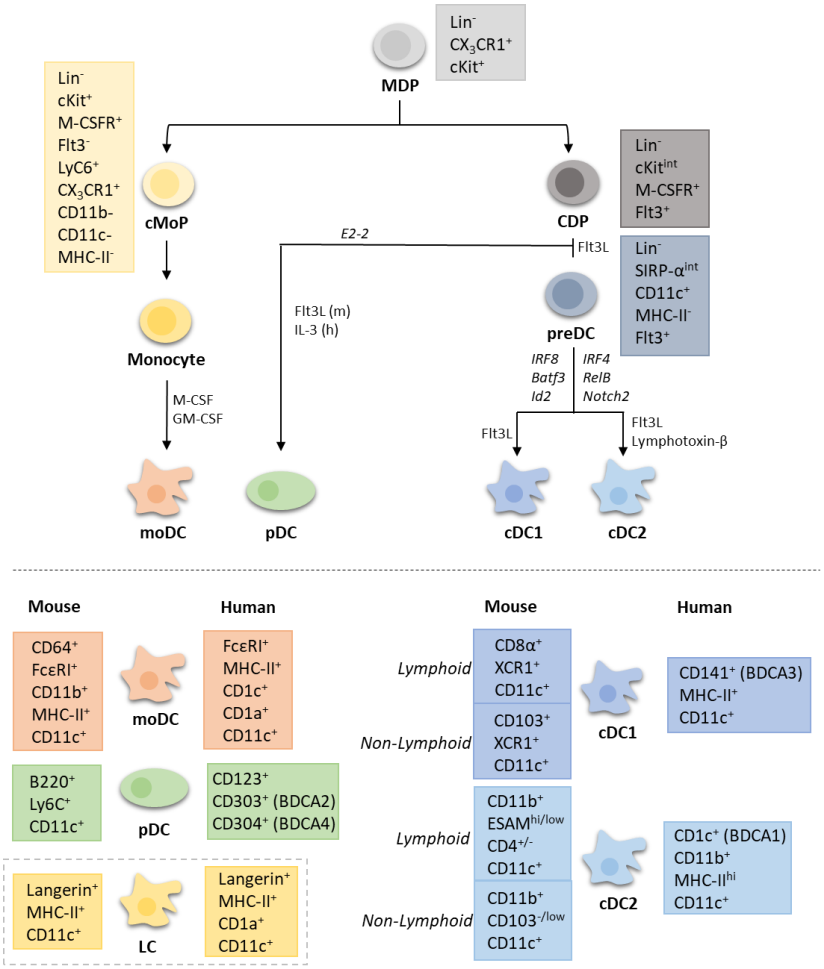

Figure 2. Overview of dendritic cell ontogeny (top) and human and mouse subtypes (bottom). Phenotypical markers are listed in boxes next to the specific cell type in corresponding colors. Cytokines and transcription factors (marked in italic) involved in cell development is listed in overview of cell ontogeny (top)**.** Abbreviations: **MDP:** Macrophage and Dendritic Cell Progenitor. **cMoP:** Common Monocyte Progenitor. **moDC:** Monocyte-derived Dendritic Cell. **CDP:** Common Dendritic Cell Progenitor**. preDC:** pre-Dendritic Cell. **pDC:** Plasmacytoid Dendritic Cell. **cDC1:** Conventional Type 1 Dendritic Cell. **cDC2:** Conventional Type 2 Dendritic Cells. **LC:** Langerhans Cells.

The story of the conventional DCs start in the mouse, where cDC1 and cDC2 are located in lymphoid and non-lymphoid tissue. While markers such as CD11c and MHC-II are expressed by both cell types, other phenotypical markers can be used to distinguish cDC1 and cDC2 cells from each other. cDC1s located in peripheral tissue express CD103 [45], while cDC1s located in lymphoid tissue express CD8α [46], and these two phenotypically different cells have similar development [47], hence both are referred to as cDC1. Independent of tissue location, both $CD103^+$- and $CD8\alpha^+$ cDC1s express XCR1 [48]. A more resent approach to distinguish between DC subtypes is based on transcriptomic analyses, which has shown that the development of different DC subtypes is dependent on a unique set of transcription factors. cDC1s are dependent on transcription factors IRF8, Id-2 and Batf3 [49-52], whereas other transcription factors are involved in the development of other DC subtypes. Finally, the environment also plays a role in DC differentiation, and cDC1 development depends on different cytokines, including Flt3L [53, 54]. The human equivalent to murine cDC1s is identified by the expression of CD141 (BDCA3) [55].

The cDC2 population is a more heterogenous, or perhaps just a more undefined, cell group. However, one general marker can be used to identify cDC2s in all tissues, namely CD11b. $CD11b^+$ cells found in non-lymphoid tissue, arising from DC restricted progenitors, are defined as $CD103^+CD11b^+$, and their development is controlled by Flt3L and GM-CSF, whereas those arising from circulating monocytes are defined as $CD103^-CD11b^+$, and their development is controlled primarily by M-CSF [56]. cDC2s found in lymphoid tissue are defined by the expression of CD11b, and the lack of CD8α, and their homeostasis is dependent on Lymphotoxin-β [57]. cDC2s found in lymphoid tissue can be further divided into two groups based on their expression of ESAM and CD4, given two cDC2 phenotypes, $CD11b^+ESAM^{hi}CD4^+$ and $CD11b^+ESAM^{low}CD4^-$ [58]. Transcription factors important for cDC2s development include: RelB, Notch2 and IRF4 [58-60]. In humans, the cells related to murine cDC2s are defined specifically by the expression of CD1c and as $CD11b^+$MHC-II^{hi} [61, 62].

The story of how pDCs was discovered is different to cDCs, since this cell type was first described in humans [63, 64]. However, research on pDC development and function was not initiated until the discovery of their murine equivalent [65, 66]. pDCs specialize in IFN-I production during viral disease, and in mice they can be identified by the expression of CD11c, B220 and Ly6C [65]. In humans, the expression of CD123, CD303 and CD304 characterizes this specific subtype, which does not express the common DC marker CD11c [61]. Their development is uniquely dependent on the transcription factor E2-2 and to some extent also on IRF4 [49, 67]. In mice, pDC development depends on Flt3L, while in humans it depends on IL-3 [68].

moDCs are found in lymphoid and non-lymphoid tissue during inflammation [69, 70] and phenotypically, they resemble DCs, which can be generated *in vitro* from

peripheral blood monocytes upon culture with cytokines, GM-CSF and IL-4 [71-73]. Their phenotype in mice is defined by the expression of CD11c, CD11b, and MHC-II, similar to cDCs, but they also express CD64 and FcεRI, making it possible to distinguish them from the cDCs [74-77]. In humans, the expression of CD11c, MHC-II, CD1c, CD1a and FcεRI, is used to identify moDCs [78, 79].

In 1868, Paul Langerhans discovered a neuron-like cell type in the epidermis of mice, later named the epidermal Langerhans Cell (LCs) [80]. Since the discovery of the DCs, LCs have been classified as part of the DC compartment, although they are often referred to as the macrophage of the DC population. One reason for this categorization is, that contrary to other DC subtypes, LCs originate from embryonic fetal liver monocytes and yolk sack hematopoiesis persisting throughout life, through self-renewal rather than through constant renewal from hematopoietic stem cells, as the other shortlived DCs [81]. Specific for murine LCs are their expression of Langerin, but as other DC subsets they also express MHC-II and CD11c. Human LCs express the same markers, and differently from the murine cells, they also express CD1a [82].

1.2.2. ANTIGEN PRESENTATION AND MATURATION

The main function of DCs is to activate the adaptive immune system. They do this through their highly developed ability to capture antigens, traveling to lymphoid tissue and presenting the captured antigens to naïve T cells, and activating these. DCs are present in non-lymphoid tissue. They are strategically located in places giving them close contact to the environment, ready to capture antigens. In lymphoid tissue, a similar location strategy applies. DCs are standing guard in the spleen as well as in lymph nodes in the marginal zone and at the subcapsular sinus, respectively, on the look-out for blood- and lymph-borne antigens [83]. When DCs are guarding their post as first line defense against antigens, they are referred to as immature DCs (iDC). iDCs have a high capacity to take up antigens, either via endocytosis or macropinocytosis [84, 85]. Since guarding their post is their most important job, they have a low expression of chemokine receptors, co-stimulatory molecules and antigen presenting molecules. When iDCs encounter a danger signal, this changes the fate of the DCs. As the name implies, danger signals are molecules able to alert the immune system of danger. Two categories of danger signals exist. Exogenous molecules, derived from infectious agents, are known as Pathogen Associated Molecular Patterns (PAMPs), and endogenous molecules derived from cancer cells, dying cells or damaged tissue, are known as Damage-Associated Molecular Patterns (DAMPs) [86, 87]. Common for these molecules is that they can bind to Pattern Recognition Receptors (PRRs), which are highly expressed on antigen presenting cells. PRRs comprise of receptors present in cell membranes and endocytic compartments, including C-Type Lectin Receptors (CLRs) as well as a wide range of Toll-Like Receptors (TLRs), and receptors present in the cytoplasm, including Nod-Like Receptors (NLRs), Rig-Like Receptors (RLRs) and AIM2-Like Receptors (ALRs). The former recognize extracellular ligands and the latter intracellular ligands [88].

Once DCs have encountered an antigen and danger signals, they transition into mature DCs (mDC), priming them to become professional antigen presenters and T cell activators. Firstly, iDCs change their expression of chemokine receptors by upregulating CCR4, CXCR4 and especially CCR7, which promotes their migration to lymphoid tissue expressing receptor ligands [89]. Further, mDCs are characterized by a decrease in phagocytotic activity, increased cytokine production and upregulation of co-stimulatory molecules, e.g. CD80 and CD86, and MHC-I and II molecules, leading to an increased ability to process and present antigens to lymphocytes [90]. All these factors play an important role in the activation of T cells, which depends on three signals being admitted by DCs. The first signal is the presentation of Ags on MHC molecules, which is recognized by specific T cells via the TCR, and this signal is essential for initiating adaptive immune responses [91]. Exogenous antigens are processed and presented via MHC-II molecules and endogenous antigens via MHC-I. However, DCs have the remarkable ability to process exogenous antigens and present them via MHC-I molecules, a process known as cross presentation. Cross presentation can occur through two different pathways, the cytosolic and the vacuolar [92]. Via the cytosolic pathway, exogenous Ags escape the phagosomes and become available for MHC-I molecules after proteasome degradation in the cytosol, followed by the transport into the ER or back into the phagosome via Transporter Associated with Antigen Processing (TAP). The vacuolar pathway is considered TAP-independent, meaning that exogenous Ags are degraded in the phagosome before loading on MHC-I molecules [92]. Upregulation of co-stimulatory molecules, CD80 and CD86, enables DCs to interact with CD28 on T cells and provides the second signal. The third signal is delivered via cytokines produced by DCs and the DC – T cell interaction is important in determining the fate of the immune response [91]. DCs polarize naïve T cells into effector T helper cells (Th cells) of different types. The categorization of Th subsets has been an area of debate and uncertainty; however, some consensus has been reached making it possible to define five major subsets, namely, Th1, Th2, Th17, Tregs and T follicular helper (Tfh) cells [93]. Lineage development of Th1 cells is initiated by the transcription factor; T box expressed in T cells (T-bet) [94]. Polarization of Th1 cells is induced by IL-12, IFN-I and IFN-γ, and their function is essential for the defense against intracellular infections and cancers, via their production of IFN-γ and TNF-β [93, 95]. The commitment to a Th2 subtype is controlled by the transcription factor GATA-3, while the cytokines involved in Th2 development include IL-4, IL-6, IL-10 and IL-11 [95, 96]. Th2 commitment leads to production of IL-4, IL-5 and IL-13. These cytokines are important for the generation of immune responses against extracellular pathogens, including parasites [93, 95]. Th17 cells particularly produce IL-17A, IL-17F and IL-22. They play a major role in the generation of autoimmune inflammation and in the generation of immune responses against extracellular bacteria and fungi [93, 95]. The orphan nuclear receptor RORγt is the main transcription factor involved in Th17 cell differentiation and IL-6, TGF-β, IL-21 as well as IL-23 promote Th17 polarization [93, 97]. Treg development is regulated by transcription factor Foxp3, and cytokines IL-2 and TGF-β [98, 99]. They produce IL-10 as well as TGF-β and can regulate immune responses

and prevent autoimmunity [100]. Thf cells represent the fifth major Th cell subset, and their development is dependent on transcription factor Bcl6 and on IL-6 or IL-21 [93, 101-103]. Tfh cells are critical for the maintenance of germinal centers, for helping and regulating B cells and for Ig class switching [101]. In immune responses against cancer, Th1 polarization is preferable and it is therefore of great interest to get DCs to produce cytokines which promotes Th1 differentiation.

1.3. TYPE I INTERFERONS AND THE STING PATHWAY

It is becoming apparent that immune responses against malignancies resembles immune responses seen during viral infections. Type I Interferons has long been known to play an important role in anti-viral immune responses [104], and thus, IFN-I is emerging as a very important player in cancer immunity.

1.3.1. TYPE I INTERFERONS

Interferons belong to a family of cytokines consisting of three subtypes. IFN-γ is the only type II IFN, while the type III IFNs consist of IFN-λ 1-4. Type I IFNs are the largest member of the family and includes IFN-α 1-13, IFN-β, IFN-ε, IFN-τ, IFN-κ and IFNω. IFN-I can affect a broad range of cells and signals upon binding to their common transmembrane receptor Interferon α/β Receptor 1/2 (IFNAR1/2) [105]. Upon binding of IFN-I, two kinases are recruited, Tyrosine Kinase (TYK2) and Janus Kinase (JAK1) [106, 107]. This recruitment leads to dimerization and phosphorylation of STAT1 and STAT2 [108-110]. STAT1/2 unites with IRF9 and forms a transcription factor complex, Interferon Stimulated Gene Factor 3 (ISGF3), which travels to the nucleus and interact with Interferon Stimulated Response Elements (ISREs) leading to the transcription of a variety of immune-regulatory genes [105].

The effector functions initiated by IFN-I affects cells involved in anti-tumor immunity. IFN-I is necessary to uphold adequate numbers of NK cells in homeostasis and is also required for elimination of tumors sensitive to NK mediated rejection [111]. IFN-I functions as a third signal for T cells, increasing their proliferation and clonal expansion as well as their capacity to produce IFN-γ and introduce cytotoxic killing [112, 113]. In addition, it stimulates the generation of memory T cells [114]. DCs increase their migratory capacity, maturation and activity via an autocrine response to IFN-I [115, 116]. Importantly, IFN-I also enhances DCs ability to cross-present Ags to T cells [117, 118]. This aptitude is essential for DCs to induce tumor rejection via T cell activation [119, 120] and highlights the desire to develop immunotherapy, which can promote DC activation and IFN-I production.

1.3.2. SIGNALING VIA STING

An important inducer of IFN-I production is the Stimulator of Interferon Genes (STING), identified in 2008 by Ishikawa and colleagues [121]. STING is a protein consisting of 379 or 378 amino acids in human and mouse, respectively, with a molecular weight of ~42 kD [121]. It contains five transmembrane domains and is located in the ER in a broad range of cells. STING activation leads to the recruitment of TANK-Binding Kinase 1 (TBK1) and the formation of a STING-TBK1 complex, leading to phosphorylation of STING [122, 123]. Once phosphorylated, the activated STING-TBK1 complex interacts with the transcription factor, Interferon Regulatory Factor 3 (IRF3), which forms an active phosphorylated dimer and translocate to the nucleus and initiate the transcription of IFN-I genes [122-124]. STING can also interact with I-Kappa Kinase α/β (IKKα/β), leading to the phosphorylation of the two molecules and the activation of the transcription factor Nuclear Factor-Kappa B (NF-κB), which in the nucleus introduce transcription of interferon- and pro-inflammatory cytokine genes, including IFN-I, TNF-α and IL-6 [124, 125]. Upon activation, new evidence indicates that STING travels from the ER and establishes its primary signaling at the ER Golgi Intermediate Compartment (ERGIC) [126, 127]. After STING has played its role at the ERGIC, it travels through the Golgi and interacts with Rab7, indicating endolysosomal degradation [128] (**Figure 3**).

The activation of STING is triggered upon the interaction with cyclic dinucleotides (CDNs). CDNs can originate from several sources; either directly from bacteria, e.g. c-di-GMP, c-di-AMP, and bacterial 3’5’-cyclic GMP-AMP (cGAMP), or from the cell itself in the form of 2’3’-cGAMP, which is generated after cytosolic DNA sensing [129-132]. Cytosolic DNA can be foreign DNA of viral or bacterial origin, or self-DNA originating from dead cells or tumor cells [124, 133]. dsDNA present in the cell cytosol is sensed by cyclic GMP-AMP synthase (cGAS) [134]. cGAS is a 60 kD enzyme, containing a nucleotidyl transferase domain, enabling the conversion of GTP and ATP into 2’3’-cGAMP as well as two DNA binding domains, binding directly to the DNA backbone, and therefore makes the recognition independent of DNA sequence [131, 134-136] (**Figure 3**).

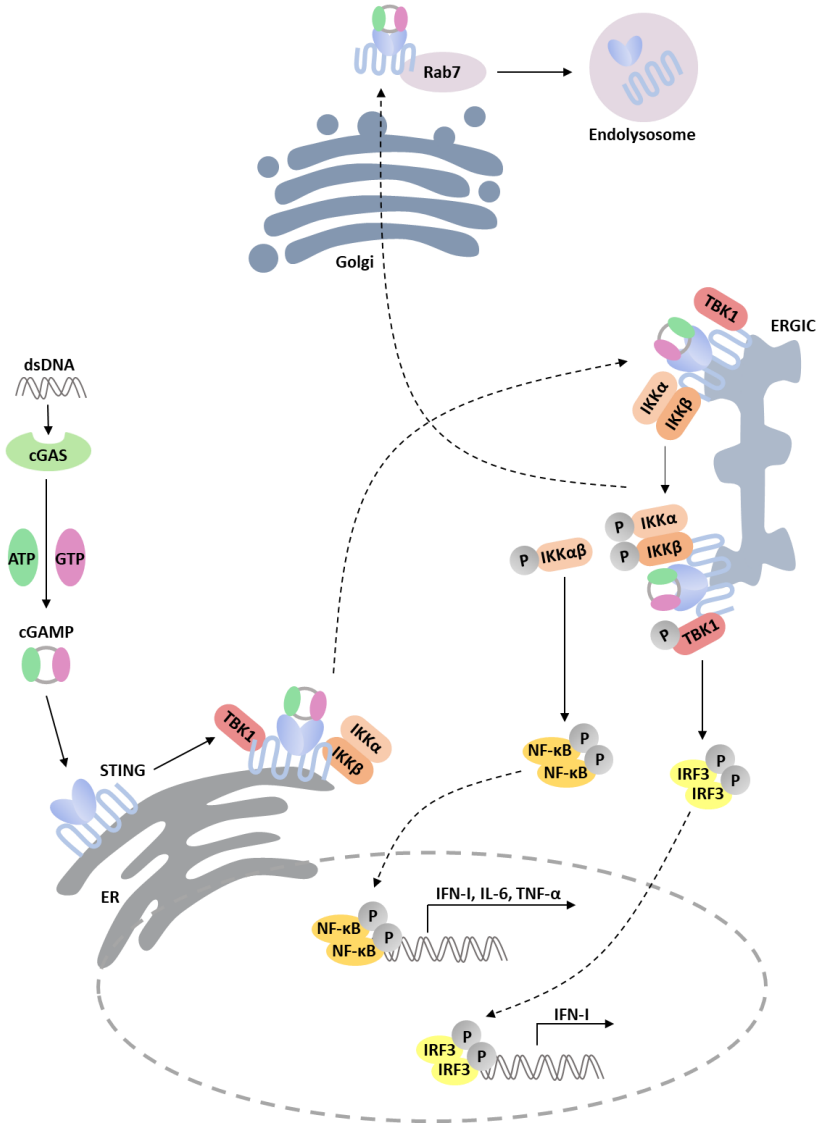

Figure 3. Overview of signaling via the STING Pathway. dsDNA in the cytosol is recognized by cGAS which catalyzes the generation of cGAMP. cGAMP is a STING agonist and binds to STING at the ER and activated STING recruits IKKαβ and/or TBK1. The complex travels to the ERGIC and after phosphorylation of IRF3 and NF-κB these two transcription factors translocate to the nucleus an induce transcription of INF-I and inflammatory cytokines. STING translocates through the Golgi and is degraded in endolysosomes in a Rab7 dependent manner. Abbreviations: **cGAS:** cyclic GMP-AMP synthase. **cGAMP:** cyclic GMP-AMP. **STING:** Stimulator of Interferon Genes. **ER:** endoplasmic reticulum. **ERGIC:** ER Golgi Intermediate Compartment.

1.4. FIGHTING CANCER WITH IMMUNOTHERAPY

The field of immunotherapy against cancer has been revolutionized during the last decade. The point of immunotherapy is to boost the patient's own immune system and lift any suppressive hold on the immune cells, leading to immune mediated elimination of tumor cells. Currently, the strategies to do this include therapy with checkpoint inhibitors, vaccines, cytokines and cellular therapy [137]. Checkpoint inhibitors is a form of therapy applying monoclonal antibodies targeted against PD-1, PD-L1 or CTLA-4, which can reverse the suppressive effects induced by the upregulation of these molecules on tumor- and immune cells in the TME [1]. Checkpoint blockade therapy, applied for treatment of several cancers, has proven safe in a range of patients, and it can improve survival when used, either as monotherapy or in combination with other therapies [138-140]. A vaccine using autologous DCs, Sipuleucel-T, has been FDA approved for treatment of prostate cancer, and it has been shown to improve patient survival [141]. Treatment with cytokines, including IL-2 and IFNα, and cellular treatment with CAR T cells have also shown promise as anti-tumor treatment [142-144]. However, with mono-treatment, the effect in many patients is still rather low, and the development of new immunotherapeutic approaches, which can be used in combinational therapy, is therefore very relevant.

1.4.1. DENDRITIC CELL-BASED IMMUNOTHERAPY

Applying DCs as vaccine formulations is an appealing method for immunotherapy against cancer. Currently, it is somewhat of a jungle to figure out how to best utilize DCs for this purpose. A well-investigated approach has been focusing on generating *ex vivo* manipulated DCs. Autologous DCs are generated from patients, either from $CD14^+$ monocytes or $CD34^+$ hematopoietic precursors and stimulated with TAs and maturation stimuli, after which they are transferred back into patients. However, this approach is both time consuming and expensive [91]. An alternative method could be to target DCs *in vivo*. DCs express a wide range of surface markers, and combining antibodies targeted towards DCs with Ags and/or maturation stimuli could elicit the activation of the DCs *in vivo* [145]. The type of DCs, which will gain access to the targeted therapy, is dependent on the choice of administration route, which can include intradermal (i.d.), subcutaneous (s.c.), intravenous (i.v.) and intraperitoneal (i.m.) delivery [146]. It is therefore important to consider the route of administration when choosing a vaccination strategy. However, it is not yet clear which administration route is the most optimal for DC targeting, and it could also be highly dependent on the specific reagent administrated.

Choosing an optimal DC target for vaccine applications has been intensely investigated and several targeting candidates have been suggested. A large group of these targets are part of the C-type lectin receptor (CLR) family. They all contain carbohydrate recognition domains, enabling them to bind to various oligosaccharides

of self and non-self-origin [147]. DEC-205 and the Mannose Receptor are both part of the type I CLR family [147]. Murine DEC-205 is expressed primarily by DCs of the mature kind, but are also found on B cells and in low expression on T cells and granulocytes [148, 149]. In humans, DEC-205 expression is high on myeloid blood DCs and monocytes, moderate on B cells and low on NK cells, granulocytes and T cells [150]. It has been shown that targeting Ags to DEC-205 in combination with anti-CD40 adjuvant elicits Ag specific T cell responses [151]. The Mannose Receptor is found primarily on macrophages, but have also been identified on iDCs and moDCs [85, 152, 153]. Targeting Ags to the Mannose Receptor results in Ag presentation on MHC-I and II molecules, initiating CD8 and CD4 T cell responses [154]. Also, included in the group of CLRs are the type II DC-specific ICAM 3-grabbing non-integrin (DC-SIGN) and Clec9A. DC-SIGN is expressed on macrophages and iDCs and, as the name implies, it is a strong binder of ICAM 3, which mediates the binding of DCs to T cells, suggesting its involvement in T cell activation [155, 156]. However, murine DC-SIGN seems functionally unrelated to human DC-SIGN, making pre-clinical testing of this molecule as a vaccine target challenging [157]. Clec9A is expressed by cDC1s and pDCs in mice, and in humans it is primarily expressed by cDC1s, but also in low amounts on B cells and a subset of monocytes [158-160]. Using Clec9A as a target for Ag delivery enriches antibody (Ab) responses and enhances CD4 and CD8 T cell responses [160]. Other targets considered in vaccine applications are the widely DC expressed molecules XCR1 and CD11c. XCR1 is expressed specifically on murine and human cDC1s [161, 162]. It is a chemokine receptor and its ligand XCL1 can be secreted by cells including NK cells and T cells, which will ensure the attraction of DCs to inflammatory areas [163]. CD11c is expressed by all subsets of murine DCs and all human DCs, except pDCs, and only low expression is found on LCs [164]. It forms a heterodimer with CD18, CD11c/CD18 (or $\alpha X\beta 2$), forming the complement receptor 4, which is a leukocyte integrin with a binding affinity for iC3b [165]. Targeting Ags to CD11c induces robust CD4 and CD8 T cell responses [166].

To generate a specific immune response against tumor cells, DCs need to meet antigens associated to or specific for the given tumor. Antigens can be delivered to DCs as short or long peptides, proteins or as whole tumor cells, and the immune response generated is dependent on the antigen epitopes available for T cell presentation [90]. In view of clinical translation, choosing the right antigen for vaccine formulations is of high importance. Antigens from tumor cells can be either tumor associated antigens or neoantigens. Tumor associated antigens (TAAs) are antigens, which are overly expressed by tumor cells, expressed only on germline cells or unexpressed in adult tissue, whereas neoantigens are tumor cell specific antigens (TSAs) generated due to tumor cell mutations [167]. TAAs are often expressed by several patients, making it easier to identify antigens that can be used in vaccine formulations. However, since TAAs are not tumor specific, it is possible that the patient will have generated a tolerance against the specific Ags. As the name implies, TSAs are tumor cell specific, but they are also often patient specific, meaning that

making a vaccine will be reliant on the screening of specific Ags from each patient [167]. Choosing the right antigen for vaccine formulations is therefore a large area of research. When generating vaccines for pre-clinical testing of target molecules, most commonly a model antigen, e.g. OVA, is incorporated in the vaccine.

To modulate and improve DC targeted vaccination, inclusion of an adjuvant is a necessity. The purpose of an adjuvant is to introduce proper DC maturation to avoid generation of tolerance, and in cancer treatment, to polarize the immune response in a Th1 direction [168]. Today, most vaccines use aluminum salts as adjuvant, which tends to induce Th2-skewed immune responses [169]. The search for adjuvants more prone to induce Th1 responses is a big part of the research on vaccine development against cancer. Currently, many of the candidates are TLR agonists, including CpG and Poly(I:C). CpG-oligonucleotides are bacterial CpG DNA, a PAMP binding to TLR9, which have been shown to induce DC maturation by increasing their expression of co-stimulatory molecules and IL-12 secretion [170]. Poly(I:C) is a synthetic dsRNA, a TLR3 agonist, and similar to CpG DNA, it induces DC maturation and Th1-skewed immune responses [171, 172]. DCs have also been shown to upregulate MHC- and co-stimulatory molecules when exposed to CD40 agonists, which could therefore also be valuable as vaccine adjuvant [173].

Several groups have investigated how targeting DCs *in vivo* can be used as anti-cancer therapy, applying different targets, antigens, adjuvants and administration routes (**Table 1**). These studies all point towards the conclusion, that utilizing DCs in this new *in vivo* vaccination setting is a valid approach for immunotherapy against cancer.

Table 1. Overview of pre-clinical studies targeting dendritic cells *in vivo* for anti-cancer therapy.

Target	Type and Outcome	Reference
LOX1	Anti-LOX1 Ab coupled to OVA Ag combined with Freund's adjuvant. S.c. administration inhibited EG.7-OVA tumor growth.	[174]
DEC-205	Anti-DEC-205 Ab coupled to OVA Ag administrated s.c. with anti-CD40 as adjuvant, inhibited MO4 tumor growth in therapeutic and prophylactic settings.	[151]
	Anti-DEC-205 Ab coupled to TRP2 Ag administrated s.c. with CpG as adjuvant, reduced the growth of established s.c. B16 tumors. Immunization with anti-DEC-205-TRP2 + CpG, inhibited growth of lung metastatic B16 tumors.	[175]
	ScFv-anti-DEC-205 coupled to gp100 Ag administrated s.c. with CpG+poly(I:C) as adjuvant, inhibited growth of established s.c. B16F10 and RET tumors.	[176]
	Immunization with i.p. administrated anti-DEC-205 coupled to HER2 Ag with poly(I:C) + anti-CD40 as adjuvants, delayed NT2.5 tumor growth and increased survival.	[177]
CD11c	ScFv anti-CD11c coupled to HER2 Ag administrated s.c. with CpG as adjuvant, protected against D2F2/E2 tumor development and inhibited D2F2/E2 tumor growth in prophylactic and therapeutic settings, respectively.	[178]
	Immunization with i.m. administrated DNA vaccine ScFv-anti-CD11c coupled to HER2 Ag prevented s.c. D2F2/E2 tumor	[179]

	development. Therapeutic vaccination in combination with low-dose cyclophosphamide (Induce Treg depletion) treatment, inhibited tumor growth and increased survival.	
Mannose Receptor	hMR Tg mice immunized i.p. with anti-hMR Ab coupled to OVA Ag with CpG adjuvant, inhibited MO-4 tumor growth.	[180]
DC-SIGN	KLH adjuvant coupled to humanized anti-DC-SIGN Ab administrated s.c. in combination with hPBMCs, inhibited RAJI tumor growth.	[181]
Clec9A	S.c. administrated anti-Clec9A coupled to SIINFEKL antigen (OVA Ag MHC-I restricted epitope) with CD40 as adjuvant, reduced development of B16-OVA-GFP lung metastasis tumors, in prophylactic and therapeutic settings.	[182]
	Anti-Clec9A coupled to MUC1 HLA-A2 epitope Ag (MUC1-A2) with anti-CD40 and poly(I:C) as adjuvant administrated s.c., inhibited growth of s.c. MC38-MUC1-A2 tumors, in prophylactic and therapeutic settings.	[183]
CD36	Immunization with s.c. administrated scFv-anti-CD36 coupled to OVA Ag, inhibited EG7-OVA tumor growth.	[184]
MHC-II	Mutated SMEZ-2 (bacterial superantigen with MHC-II binding capacity) coupled to OVA Ag administrated i.v. with α-GalCer as adjuvant, delayed established s.c. B16-OVA tumor growth.	[185]
Bst2	Mice immunized i.p. with an anti-Bst2 Ab (pDCs specific targeting) coupled to OVA Ag in combination with poly(I:C) as adjuvant, showed delayed tumor growth and increased survival in s.c. B16-OVA tumor model.	[186]
XCR1	Different immunization regimes with DNA vaccination of mXcl1 (XCR1 ligand) coupled to Ag GPC3, obstructed development and growth of i.p. administrated Diethylnitrosamine (DEN) induced liver tumors in mice.	[187]
	Prophylactic and therapeutic i.d. vaccination with DNA vaccine XCL1 coupled to OVA Ag slowed B16-OVA tumor growth.	[188]

1.4.2. STING ACTIVATION AND IMMUNOTHERAPY

STING is an essential initiator of IFN-I production, and STING agonists have been investigated as adjuvant in cancer therapy. The flavonoid compound, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), is a direct agonist of murine STING (mSTING), but it does not bind to human STING (hSTING) [189-191]. It has anti-tumor activity in murine tumor models, where activation of NK cells and macrophages, priming of T cells and induction of tumor rejection is observed and found to be STING dependent [192, 193], proving that the use of direct STING agonists could be a new addition to cancer therapeutics. Based on this, using the known hSTING agonist 2'3'-cGAMP for the same purpose has been tested and proved to reduce tumor load in combination with radiotherapy [194]. However, 2'3'-cGAMP and other CDNs are sensitive to enzymatic degradation and hold low membrane diffusion capacities due to their anionic nature [195]. A synthetic form of cGAMP has been developed to try and circumvent these issues. By altering the structure of phosphate linkages and substituting oxygen atoms with sulfur atoms, this synthetic form of cGAMP, ML RR-S2 CDA (Named: ADU-S100), is more resistant

to enzymatic degradation and has enhanced binding to hSTING. Furthermore, it enhances expression of STING associated cytokines, e.g. IFN-β, IL-6, TNF-α, and has increased anti-tumor efficacy after intratumoral (i.t.) injection in murine tumor models [193]. The proof of an advantage of direct STING agonists as anti-tumor therapy has sprung a new line of therapeutic attempts utilizing CDNs. A whole-cell vaccine, made of irradiated GM-CSF secreting tumor cells (Named: STINGVAX), applied ADU-S100 as adjuvant, and proved to have enhanced anti-tumor efficacy [196]. Other therapeutic approaches investigate the use of CDNs, in combination with different carrier systems, ranging from liposomal carriers [197-200] and other types of micro/nanoparticle carrier systems [201-205], to incorporation in hydrogels and biopolymers [206, 207], and the development of new direct STING agonists [208-210] (**Table 2**). Having the ability to either work alone, as adjuvant in vaccines or in combination with other types of immunotherapy, e.g. check point inhibitors or radio- and chemotherapy, the generation of STING stimulating agents will likely be an important addition in the immunological fight against cancer.

Table 2. Overview of pre-clinically tested STING activating anti-cancer therapy

Name	Type and Outcome	Reference
2'3'-cGAMP	Natural direct STING agonist. I.t. administrated of 2'3'-cGAMP in combination with radiotherapy, reduced tumor load in established s.c. MC38 tumors.	[194]
DMXAA	Direct STING agonist 5,6-dimethylxanthenone-4-acetic acid (DMXAA). I.t. administration of DMXAA in established s.c. B16.SIY tumors led to tumor regression. I.v. administration prolonged survival of mice with established C1498.SIY ALM tumors.	[193, 211]
ADU-S100	Direct STING agonist, ML RR-S2 CDA, synthetic 2'3'-cGAMP. I.t. administration reduced tumor growth and improved overall survival in established s.c. B16F10 tumors.	[193]
STINGVAX	Lethally irradiated GM-CSF-expressing tumor cells mixed with ADU-S100. Contralateral administration of STINGVAX in combination with anti-PD-L1, reduced tumor load in established s.c. B16 tumors and induced complete tumor regression in established s.c. CT26 tumors.	[196]
NP-cdGMP	Liposomal nanoparticle carrier containing cyclic di-GMP. Enhanced uptake of CDN in lymph nodes. S.c. administration in combination with antigen peptide vaccination, reduced tumor development and prolonged survival in established s.c. EG.7-OVA and B16 tumors.	[197]
c-di-GMP/ YSK05	Liposomal nanoparticle carrier containing cyclic di-GMP. Immunization with s.c. administration in combination with OVA Ag vaccination, prohibited development of s.c. EG.7-OVA tumors. I.v. administration inhibited tumor growth in B16F10 lung metastatic melanoma tumors.	[198, 199]
PEG Liposomal cGAMP	PEGylated liposome carrier containing 2'3'-cGAMP.	[200]

	I.v. administration in combination with anti-CTLA-4 and anti-PD-1, reduced tumor size in B16F10 lung metastatic melanoma tumors.	
CAR T cell/ STING agonist Implant	Biopolymer containing CAR T Cells and cyclic-di-GMP. Biopolymer complex implanted onto the tumor surface of orthotopic pancreatic KPC tumors, induced tumor regression and increased survival. Implanted into resection site of surgically removed B16F10 tumors, delayed tumor relapse.	[206]
PC7A NP	Synthetic polymeric nanoparticle. Direct STING activator. S.c. administration reduced tumor growth in established B16-OVA, B16F10, MC38 and TC-1 tumors and increased survival in B16-OVA and TC-1 tumor models. In combination with radiotherapy, eradication and prevention of relapse were observed in TC-1 and B16-OVA tumors, and survival was increased.	[208, 209]
STINGel	Hydrogel containing ADU-S100. S.c. administration decreased MOC2-E6E7 tumor growth and increased survival.	[207]
cGAMP-NP	Nanopartical containing 3’3’-cGAMP. I.v. administration reduced tumor growth and prolonged survival in established orthotopic C3(1)Tag and B16F10 tumors.	[201]
diABZI	Linked amidobenzimidazole. Direct STING agonist. I.v. administration inhibited tumor growth of established CT26 tumors and prolonged survival.	[210]
Ace-DEX cGAMP MP	Acid-sensitive acetalated dextran microparticle containing 3’3’-cGAMP. I.t. administration inhibited B16F10 tumor growth and prolonged survival.	[204]
STING-NP	Polymer nanoparticle carrier containing 2’3’-cGAMP. I.t. and i.v. administration +/- combination with anti-CTLA-4 and anti-PD1, inhibited established B16F10 tumor growth, prolonged survival and enhanced protection against tumor recurrence.	[205]

CHAPTER 2. OBJECTIVES

The overall aim of this thesis was to find new approaches to target and enhance activation of the STING pathway, and to evaluate if these approaches could be applied as anti-cancer immunotherapy. This was done through two main studies.

Study I: The first study investigated whether a dendritic cell-targeted adjuvant could lead to a STING-dependent cell activation and function as immunotherapy.

This was investigated through the study described in *Manuscript I* (*Appendix I*) and supported by a focused research review described in *Review I* (*Appendix II*).

The research questions were:

- Can dsDNA, delivered to the cytoplasm of dendritic cells, induce maturation?
- Can CD11c be used as a target for delivery of dsDNA to dendritic cells?
- Can dendritic cells, matured with dsDNA, activate T cells?
- Can the human monocytic cell line THP-1 be used as a DC maturation model?
- Is dsDNA induced THP-1-DC maturation dependent on STING and cGAS?

Study II: The second study investigated whether cholesterol depletion, induced by a variety of cholesterol inhibitors, e.g. Filipin-III, MβCD and Nystatin, could prime STING activity and function as anti-cancer therapy.

This was investigated in the study described in *Manuscript II* (*Appendix III*).

The research questions were:

- Can cGAMP be used to activate human and murine macrophages and dendritic cells?
- Can cholesterol depletion prime cGAMP induced STING activation?
- Do membrane cholesterol levels influence the activity and localization of STING?
- Can cholesterol depletion be applied as anti-cancer therapy?

CHAPTER 3. RESULTS

The main findings from the studies are summarized here. Detailed results are described in the manuscripts (*Manuscript I, Review I and Manuscript II, Appendix I-III*).

3.1. STUDY I

Boosting the effect of tumor specific T cell responses in cancer patients has proven to be an interesting approach for anti-cancer treatment. Activation of DCs *in situ* may aid the generation of tumor specific T cell responses. dsDNA has been shown to be able to activate DCs *in vivo* [133]. dsDNA interacts with the cytosolic DNA sensor cGAS and leads to the activation of the STING pathway, which promotes IFN-I gene transcription [131, 134]. IFN-I production is important for immune responses against viral infections, and its importance in anti-tumor immune responses is becoming increasingly evident. Targeting DCs with a strong maturational stimulus to enhance their activation, and hence their ability to activate tumor specific T cells, could potentially be a way to improve the efficacy of immunotherapy, which is currently applied as cancer treatment.

We investigated how moDCs and THP-1 cells would respond to the delivery of targeted and untargeted dsDNA. We delivered dsDNA to moDCs using the transfection reagent lipofectamine and observed that dsDNA was delivered to the cell cytoplasm, which increased DC maturation. Similarly, targeting dsDNA to CD11c led to the internalization of the dsDNA and induced DC maturation. DCs matured with dsDNA had the ability to increase T cell proliferation in a multiple leukocyte reaction. THP-1 cells cultured with GM-CSF, IL-4 and ionomycin induced the generation of DC-like cells. We applied THP-1 cells as a model for DC maturation and observed that expression of maturation markers was increased upon delivery of dsDNA. Further, we observed that maturation induced by dsDNA was abolished in cGAS and STING KO cells (*Manuscript I, Appendix I*) [212].

Collectively, these results and results previously done in our group, investigating DC targeting *in vivo*, are summarized in *Review I* (A*ppendix II*) [213] and based on this, we believe that targeting dsDNA to DCs via CD11c could potentially be applied as immunotherapy against cancer by activating DCs in a STING dependent manner.

3.1. STUDY II

IFN-I is emerging as an important player in anti-cancer immune responses. Therefore, developing therapy targeting the STING pathway, which leads to enhancement of IFN-I production, is of high interest. STING can be directly activated by CDNs including 2'3'-cGAMP, which is the product generated upon the activation of cGAS after the recognition of cytosolic DNA.

We investigated whether cGAMP could induce the activation of human and murine DCs and macrophages *in vitro*. The cells were stimulated with cGAMP alone or in combination with transfection reagent lipofectamine. We observed that high concentrations of cGAMP alone was able to induce maturation of both human and murine DCs, and in combination with lipofectamine, low concentrations of cGAMP also activated human and murine macrophages. We discovered that pre-treatment of human and murine DCs and macrophages, with cholesterol inhibitor Filipin-III, primed cells to respond to lower concentrations of cGAMP, and thus increased their activation. To confirm that these observations were in fact due to the cholesterol inhibition, and not other adverse effects induced by Filipin-III treatment, we utilized another cholesterol depletion reagent, MβCD, and observed that this reagent also primed DC maturation and activation of macrophages, in response to cGAMP. We observed that Filipin-III and MβCD priming was dependent on enhanced activation of the STING pathway, in which phosphorylated STING and TBK-1 was upregulated. Addition of cholesterol to THP-1 cells retained STING in the ER and completely abolished phosphorylation of STING and TBK1, confirming that cholesterol levels are a key regulator of STING activity. In a murine tumor model, i.t. treatment with MβCD in established tumors inhibited tumor growth as long as treatment was maintained. Lastly, we tested the clinically relevant cholesterol depletion reagent Nystatin as pre-treatment before cGAMP stimulation and saw that this reagent also enhanced DC maturation, compared to cGAMP treatment alone (*Manuscript II, Appendix III*).

Collectively, these results show that cGAMP-induced activation of DCs and macrophages can be enhanced by pre-treatment with several different cholesterol depleting or blocking reagents, and that this activation is dependent on increased activation of the STING pathway. The depletion of cholesterol increases STING sequestering from the ER and provides protection against tumor growth in a murine tumor model. We suggest that application of Nystatin or other clinically approved cholesterol inhibitors will aid the immunological treatment of cancers via the enhancement of STING pathway activation (*Manuscript II, Appendix III*).

CHAPTER 4. DISCUSSION

4.1. METHODOLOGICAL CONSIDERATIONS

The first part of the discussion will be on methodological considerations. Special weight will be put on the model systems, applied to evaluate DC biology in experimental settings. Included is a discussion on the use, and choice, of the murine tumor model used in experiments. A closer look will be taken on the therapeutic strategies applied in the studies.

4.1.1. MONOCYTE-DERIVED DENDRITIC CELLS

DCs only constitute approximately 0.2 % of peripheral blood leukocytes and approximately 2 % of mononuclear blood cells in healthy adults [214]. This makes it challenging to obtain high numbers of cells in experimental settings to study DC biology. Protocols have been developed to generate DCs *in vitro* from peripheral blood $CD14^+$ monocytes or $CD34^+$ hematopoietic stem cells [215]. $CD34^+$ cells can be isolated from bone marrow, cord blood or peripheral blood and be induced to differentiate into DCs during 12-14 days in cultures containing GM-CSF and TNFα [216-219]. These protocols generate $CD1a^+$ DCs, which also express MHC-II, CD80, CD86, CD83 and CD40, and have the ability to stimulate T cells. However, the number of DCs generated from these cultures are typically low, 10-40 % [216-219]. The low accessibility to obtain cells, particularly from bone marrow and cord blood, together with the low DC numbers, makes it preferable to use other protocols for DC generation. In our studies (*Manuscript I and II, Appendix I and III*) [212], we applied moDCs generated from $CD14^+$ blood monocytes, based on the protocol described by Sallusto et al. [71]. This protocol which is extensively used for *in vitro* DC generation, utilizes $CD14^+$ monocytes derived from peripheral blood, cultured for 6-7 days in GM-CSF and IL-4. This protocol generates a homogenous population of $CD1a^+CD11c^+$ DCs, with low expression of MHC-II, CD80/86 and CD83, resembling iDCs and with a yield of up to 90 % [71, 220, 221]. Maturation can be induced upon addition of LPS or TNFα, leading to upregulation of MHC-I and II, CD80, CD86, and CD83 and an increase in T cell stimulatory capabilities [71, 220, 221]. This method is very usable for *in vitro* studies of DCs biology, specifically for studying DC maturation, due to the relatively high availability of blood monocytes. In addition, it has been shown in mouse tumor models, that the effect of anti-tumor therapy and proper $CD8^+$ T cell activation is dependent on the presence of moDCs [222, 223]. Therefore, using moDCs in *in vitro* experiments provides a very useful and clinically relevant method for studying DC maturation and to test therapy targeting DCs.

4.1.2. BONE MARROW-DERIVED DENDRITIC CELLS

In mice, it is also challenging to isolate high numbers of DCs directly from blood, and the most common way to obtain large numbers of DCs is to generate them from precursors of the bone marrow compartment. As described by Inaba et al. [224], it is possible to generate large DC numbers by cultivating bone marrow cells with GM-CSF. A modified protocol also includes IL-4 in the cultures [225]. Independent of the protocol, the cells that are generated express MHC-II, CD80 and CD86. However, the addition of IL-4 induces cells to have a higher expression of these markers compared to cells cultured with GM-CSF alone, and they also have a higher ability to stimulate T cells [225]. A third approach to generate BMDCs applies Flt3L to induce differentiation [226]. In our study (*Manuscript II, Appendix III*), we generated BMDCs in IL-4 and GM-CSF cultures. Although compared to BMDCs differentiated with GM-CSF only, these cells show a more mature baseline, addition of maturation stimuli, e.g. TNFα, LPS or CpG, enhance cell maturation, confirming their use as a model for maturation [227]. Generating BMDCs with Flt3L produces a heterogeneous cell population containing pDC-, $CD8^+$ cDC- and $CD8^-$ cDC equivalents, while BMDCs generated with IL-4 and GM-CSF gives a more homogeneous cell population of myeloid $CD11b^{high}$ cells [228]. IL-4 and GM-CSF generated BMDCs show superior ability to stimulate T cells, compared to Ftl3L generated BMDCs, and they resemble inflammatory DCs, hence moDCs [227, 228]. Application of BMDCs as vaccination, in a murine tumor model, shows that IL-4 and GM-CSF generated BMDCs provide superior protection compared to Flt3L generated BMDCs, an observation that could be explained by the Th2-skewed T cell responses induced by Flt3L generated BMDCs [227]. IL-4 and GM-CSF generated BMDCs are therefore a good model for maturation studies as well as for investigations of anti-tumor therapy, and results from experiments using IL-4 and GM-CSF BMDCs should be comparable to results obtained using our model for the generation of human moDCs (*Manuscript I and II, Appendix I and III*) [212].

4.1.3. THP-1 MATURATION MODEL

In order to investigate the involvement of STING and cGAS in DC maturation, we used a model based on the human monocytic THP-1 cell line (*Manuscript I, Appendix I*) [212]. The main advantage of applying a cell line model for these studies was that we could generate stable gene knock out of cGAS and STING, using CRISPR/Cas9 gene editing technology. By using a cell line model, it is easy to obtain large amounts of cells, and furthermore the cell homogeneity is high, while donor-to-donor variability is avoided, resulting in more reproducible results [229].

The THP-1 cell line was derived from the blood of a boy with acute monocytic leukemia and established as a leukemic cell line characterized with monocytic properties having immunological functions [230]. THP-1 cells cultured with Phorbol-12-myristate-13-acetat (PMA) generate cells resembling macrophages derived from

peripheral monocytes, and this model has been extensively used to study the biology of macrophages [231]. We therefore applied this model to investigate macrophage activation in study II (*Manuscript II, Appendix III*). Using the THP-1 cell line as a model to study DC biology is less well investigated. However, studies have indicated that it is possible to use THP-1 cells to generate cells resembling moDCs. Berges and colleagues [229] applied GM-CSF and IL-4 to induce differentiation of THP-1 monocytes into an immature-like DC, having high endocytic activity. Culturing of the immature-like DCs for 3 days in GM-CSF, IL-4, TNFα and ionomycin, induced them to differentiate into a mature-like DC, shown by decreased endocytic activity, upregulation of HLA-DR and co-stimulatory molecules, which increased the ability to activate T cells [229]. Inspired by this, we generated a protocol where we cultured THP-1 monocytes in IL-4, GM-CSF, ionomycin as well as dsDNA, and saw that we could generate a mature DC-like phenotype (**Figure 4**). The cells expressed maturation markers CD86 and HLA-DR as well as the DC specific marker DC-SIGN, and contained high levels of IL-12 mRNA (*Manuscript I, Appendix I*) [212].

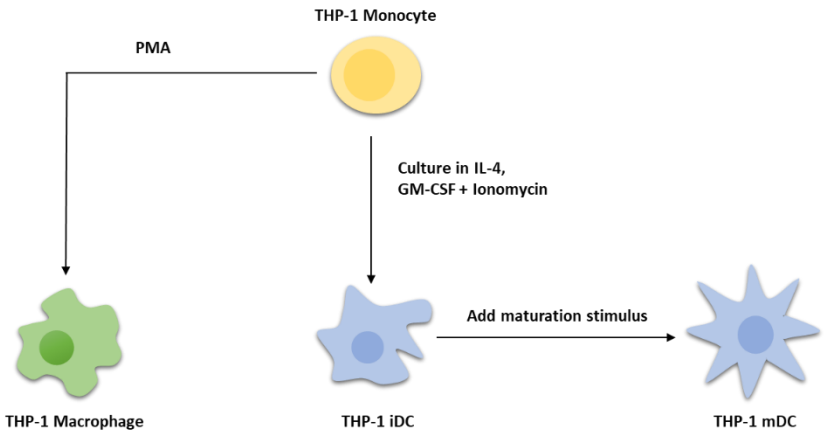

Figure 4. THP-1 cell line models. Monocytic leukemia THP-1 cells (THP-1 Monocytes) cultured in medium supplemented with IL-4, GM-CSF and Ionomycin for 3 days differentiate into immature DC-like cells (THP-1 iDC). Addition of maturation stimuli induced cells to differentiate into mature DC-like cells (THP-1 mDC). THP-1 monocytes differentiated into macrophage-like cells (THP-1 Macrophage) in the presence of PMA.

Berges et al. [229] also showed that the myelogenous leukemia $CD34^+$ cell line KG-1 had potential to develop into DC-like cells, and this has been supported by other studies [232, 233]. CD1a is a marker used to identify human myeloid DCs with capacity to polarize a Th1 response and produce high levels of IL-12 [234]. A disadvantage with THP-1 and the KG-1 DC models are that the number of $CD1a^+$ DCs is rather low [235]. The MUTZ-3 cells, a $CD34^+$ acute myeloid leukemia cell line, are an immortal equivalent to $CD34^+$ DC progenitors and can, similar to THP-1 and KG-1 cells, differentiate into DCs upon culture in GM-CSF, IL-4 and TNFα [235]. Applying this model generates higher numbers of $CD1a^+$ DCs, and this model

is being increasingly used to study DC biology, specifically since the cells generated have been shown to resemble *in vitro* generated moDCs [236]. Although MUTZ-3 cells are becoming a highly validated model for $CD34^+$ derived DCs, yielding quite high numbers of $CD1a^+$ DC-like cells, we showed in our studies that THP-1 cells could also serve as a model for DC maturation [212]. The THP-1 cell line is, different from KG-1 and MUTZ-3 cells, a cell line with monocytic origin, and is able to differentiate into both DC- and macrophage-like cells, making this model very useable for our studies, where we study both of these cell types (*Manuscript I and II, Appendix I and III*) [212].

4.1.4. MURINE TUMOR MODELS

Testing immunotherapy *in vitro* gives a good indication of how specific cells, e.g. DCs, will respond to the therapy. However, to evaluate how therapy will function in an *in vivo* system and making results translational before clinical testing, it is necessary to test efficacy in murine tumor models, and a variety of different models have been developed for this purpose. The choice of a model is usually built on an estimation of the advantages and disadvantages associated with the user-friendliness of the model and an estimation of how translatable the results will be. In our study (*Manuscript II, Appendix III*), we applied the MC38 syngeneic tumor model. The clear advantage of this type of model is that it is easy to use and highly reproducible, but unfortunately, it lacks the heterogeneity seen in human cancers [237]. A variety of synergetic tumor models exists and there is a high diversity in the way they respond to immunotherapy [238, 239]. The MC38 tumor model has been shown to be highly immunogenic and when combinational treatment, including immunotherapy with anti-PD-L1, is applied, significant tumor reduction is seen, while this is not the case when the same treatment is applied in non-immunogenic tumor models [239]. This difference in response to immunotherapy seen in the mice models highlights the importance of determining whether tumors in patients are immunogenic or non-immunogenic, in order to better predict if the treatment will have an effect. Using immunotherapy for cancer treatment will likely function best when applied for treatment of immunogenic tumors. Testing immunotherapy in immunogenic tumor models, e.g. MC38, as we did in our study (*Manuscript II, Appendix III*), is likely the best way to evaluate the efficacy of this type of treatment. However, applying a combination of immunotherapy, targeting several parts of the TME and the immune system, could potentially turn non-immunogenic tumors into immunogenic tumors. We evaluated the efficacy of the treatment by following tumor growth in the mice. Including analysis of the TME before and after treatment, to investigate the amount of infiltrating T cells, might aid to evaluate the effect of the treatment.

4.1.5. THERAPEUTIC STRATEGIES

In our study (*Manuscript I, Appendix I*) [212],we wanted to investigate if DNA could be used to induce DC maturation, with the goal of applying this as a therapeutic strategy for cancer treatment. DNA is a DAMP, which can be released from dying cells or originate from pathogens, and activate the STING pathway via the cytosolic DNA sensor cGAS. Extracellular DNA, derived from tumor cells, can enter the cytoplasm of APCs *in vivo*, through a yet unknown mechanism [133]. We saw in our study that uptake of pure DNA by DCs was limited (*Manuscript I, Appendix I*) [212]. The reason for the low uptake of extracellular DNA is due to a high rate of enzymatic degradation and the net negative charge of DNA, which limits its access through the negatively charged cell membrane [240, 241]. Lipofectamine is a reagent, which form complexes with DNA and enhances its entry into cells via cationic lipids [241, 242]. We chose to apply lipofectamine to deliver DNA to DCs and THP-1 cells (*Manuscript I, Appendix I*) [212]. We observed that lipofectamine was able to mediate the delivery of DNA to cells. However, we also observed enhanced maturation of lipofectamine only treated cells (*Manuscript I, Appendix I*) [212]. The increased maturation of DCs upon lipofectamine treatment could be due to the cell perceiving the membrane disruption as a danger signal [243].

We have previously shown in our group [244, 245], that CD11c functions as a target for antigen delivery to DCs *in vivo* (*Results summarized and discussed in Review I, Appendix II*)[213], and based on this, we wanted to investigate if this receptor could also be used to deliver DNA to DCs (*Manuscript I, Appendix I*) [212]. We applied a conjugation strategy, using succinimidyl 4-formylbenzoate (4SFB) and succinimidyl 6-hydrazinonicotinate acetone hydrazine (Hy-Nic) modifications of DNA and Ab, respectively, leading to covalent binding of Ab and DNA, and generation of the Ab-DNA construct (**figure 5**) [246]. This method resulted in high amounts of Ab-DNA conjugates, binding one or two DNA molecules per Ab, which could be internalized by cells, confirming that the Ab did not lose function after conjugation (*Manuscript I, Appendix I*) [212]. Whether the Ab gets conjugated with one or two DNA molecules does not affect Ab function. Constructs generated with the described conjugation technique are stable for long periods under physiological conditions [246]. This validates this method as an excellent way to generate Ab-DNA conjugates, which can be used in a variety of biological assays.

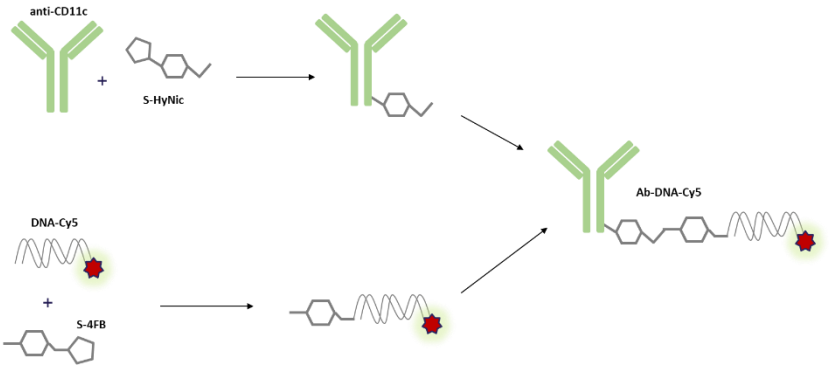

Figure 5. Conjugation of anti-CD11c antibody and dsDNA. dsDNA 5'-Cy5 labeled and modified with a 3'-amine group was coupled to succinimidyl 4-formylbenzoate (S-4FB) and anti-CD11c antibody was coupled to succinimidyl 6-hydrazinonicotinate acetone hydrazine (S-HyNic), generating the conjugation via a covalent linkage.

Similar to DNA, cGAMP is vastly hydrolyzed in the extracellular compartment and has low membrane diffusion capabilities due to its anionic nature [195]. Therefore, we applied lipofectamine to deliver cGAMP to cells *in vitro* (*Manuscript II, Appendix III*). This approach resembles methods used in *in vivo* studies applying liposomal carrier systems for delivery of cGAMP [197-200]. However, when applying these carrier systems for cGAMP delivery *in vivo*, extensive preparation is necessary. In cell membranes cholesterol functions as a central lipid, and depletion of cholesterol can be used to study membrane permeability and cellular trafficking [247, 248]. Therefore, manipulating the cell membrane's content of cholesterol might be a way to enhance entry of cGAMP and modulate cellular trafficking, which could influence STING activation. In our study (*Manuscript II, Appendix III*), we investigated how cholesterol depletion effected cellular responses to cGAMP.

Filipin-III is a polyene macrolide antibiotic, which forms complexes with sterols in aqueous solutions and forms aggregates in lipid membranes [249, 250]. It also has an affinity for phospholipids and causes membrane instability, without affecting membrane resistance [251, 252]. The effects Filipin-III has on cellular membranes makes it cytotoxic, and it is therefore not used for clinical applications. However, due to its fluorescent properties, it is often used as a fluorescent probe for cholesterol staining [249]. It is a highly validated method to apply Filipin-III for cholesterol inhibition in membranes and in our study (*Manuscript II, Appendix III*), we therefore used Filipin-III to make a prove-of-concept. However, it is likely that Filipin-III treatment is associated with adverse effects, and we therefore tested another well-known cholesterol depletion reagent, MβCD, to confirm that our observations were due to depletion of cholesterol and not to side effects induced by Filipin-III (*Manuscript II, Appendix III*). Cyclodextrins are cyclic oligosaccharide, and the addition of methyl groups increases the reagents solubility [248]. Cholesterol

depletion mediated by MβCD occurs because MβCD forms a cone, in which cholesterol is complexed into the central cavity, in a MβCD:Cholesterol 2:1 complex [253]. Applying MβCD for cholesterol depletion induced results similar to what was observed when applying Filipin-III, confirming that the results were due to cholesterol inhibition (*Manuscript II, Appendix III*). An advantage of using MβCD is that it can be applied for *in vivo* studies, which we also utilized in our study (*Manuscript II, Appendix III*). However, MβCD is not approved for clinical use, so we wanted to find a cholesterol depletion reagent which was clinically approved to confirm our results and make them translatable. Amphotericin β and Nystatin are both, similar to Filipin-III, included in the family of polyene macrolide antibiotics, but are approved for clinical use to treat fungal infections [249, 250]. Amphotericin β and Nystatin interact with sterols. However, Nystatin has low association with cholesterol compared to both Filipin-III and Amphotericin β, and due to the lower association with cholesterol, Nystatin induces less damage to cell membranes [250]. This made Nystatin the preferable clinically relevant reagent to use in our studies (*Manuscript II, Appendix III*). In addition, Nystatin does not affect membrane stability, but reduces membrane resistance and enhances anion permeability. These are features which will likely enhance cGAMP entry to cells without inducing toxicity [252].

4.2. GENERAL DISCUSSION

DC, IFN-I and hence, the STING pathway, are all vital components of innate immunity, and strong innate immunity is associated with better adaptive anti-tumor immunity [254]. For this reason, utilizing immunotherapeutic approaches to enhance DC- and STING pathway activity and promote IFN-I production could be a beneficial addition to current treatment options. The second part of the discussion will be focusing on DC targeting as well as DC maturation and activation of the STING pathway in view of what is learned from our studies.

4.2.1. TARGETING DENDRITIC CELLS

Targeting DCs directly *in situ* with Ags with and without adjuvants serves as an interesting approach for immunotherapy against cancer and has been shown to promote anti-tumor responses in several pre-clinical studies (**Table 1**). Considerations of the choice of target, which stimuli should be delivered and whether combination with other treatment options should be applied, are important to find the most optimal way to activate DCs with this approach. In our study (*Manuscript I, Appendix I*), we chose to target a maturation stimulus, dsDNA, to DCs via CD11c, and analyzed how this affected DC maturation [212]. The choice of target was based on considerations summarized and discussed in *Review I* (*Appendix II*) [213]. We found that CD11c would be an interesting target, based on previous studies made in our group, where several DC surface molecules were screened, in order to evaluate their prospective as targets for Ag delivery (*Review I, Appendix II*) [213, 245]. Our previous results showed that targeting Ags to CD11c induced high numbers of IFN-γ producing T cells

and robust Ab responses (*Review I, Appendix II*) [213, 244, 245]. The benefits of using CD11c as a target have been confirmed in other studies, showing that targeting Ags to CD11c enhance T cell responses [166]. In mice, CD11c is considered a highly DC specific target, while in humans it is expressed substantially on DCs, but is also expressed on other immune cells [164]. The expression pattern of CD11c in mice makes it an interesting target due to its high DC specificity. However, it is not necessarily a disadvantage that human expression of CD11c is seen on a broader range of immune cells, including macrophages and monocytes, since activation of a broader spectrum of immune cells might enhance anti-tumor responses induced by targeted immunotherapy.

Vaccination targeting DCs to introduce anti-tumor responses should contain an adjuvant, which introduces DC maturation, to promote immunogenicity and activation of cytotoxic T cells, rather than promoting tolerance [145]. DAMPs and PAMPs are recognized by PRRs, including TLRs, RLRs, NLRs, CLRs, ALRs and cytosolic DNA sensors, expressed by DCs, and recognition of DAMPs and PAMPs lead to DC maturation [88]. In the 1890's William B. Coley, demonstrated that the delivery of bacterial products, which contains TLR agonists, to cancer patients, promoted innate immune responses and induced tumor regression [254]. Imiquimod and Resiquimod are TLR7 agonists, approved for topical treatment, which can increase pDC maturation and IFN-I production, and Imiquimod has been shown to skew immune responses towards a Th1 direction [255, 256]. Treatment with Imiquimod in a cutaneous breast cancer model is associated with increased infiltration of $CD11c^+$, $CD4^+$ and $CD8^+$ cells in tumors as well as a significant inhibition of tumor growth. Furthermore, the effect of Imiquimod treatment is enhanced by combining it with radio- or chemotherapy [257]. Similarly, it has been shown that combinational treatment with a TLR3-specific adjuvant, which promotes IFN-I production, and anti-PD-L1, induce Th1-directed immunity in tumors and increase $CD8^+$ T cell- and DC populations [258]. Additionally, topical treatment with Imiquimod has proved to be safe and beneficial for combinational treatment in breast cancer patients [259]. This highlights the benefits of applying strong adjuvants as anti-cancer treatment to promote IFN-I production and DC maturation, leading to enhanced T cell activation, and the CD11c targeted DNA generated in our study (*Manuscript I, Appendix I*)[212], functions as an adjuvant, holding these properties. In addition, we observed that the increased DC maturation was dependent on activation of the STING pathway (*Manuscript I, Appendix I*) [212]. Targeting DNA to DCs to enhance maturation and increase their ability to promote tumor specific T cell responses may be a new approach to induce immunological clearance of tumor cells (*Review I, Appendix II*) [213], and finding ways to increase activity of the STING pathway might aid the process further.

4.2.2. PRIMING THE STING PATHWAY

DCs are able to initiate adaptive immune responses and are important for the generation of anti-tumor immunity. Activation of the STING pathway leads to enhanced IFN-I production and it has been proved through a variety of studies that STING agonists can lead to inhibition of tumor growth and prolong survival (**Table 2**). Furthermore, IFN-I increases DC maturation, migration and their ability to activate T cells [260].

In our studies, we applied two different approaches to activate the STING pathway. In *Study I*, we activated DCs by delivering DNA via CD11c (*Manuscript I, Review I, Appendix I-II*) [212]. Several cytosolic DNA sensors have been identified, including DAI, DDX41, AIM2 and IFI16 [261, 262]. However, knock out of DAI and IFI16 in several different cell types does not alter IFN-I production in response to DNA, proving that these DNA sensors are not essential for IFN-I production [263, 264]. Contrary to this, knock out of cGAS makes both pDCs and cDCs from mice unable to produce IFN-I and severely lowers IFN-I production *in vivo* in viral infection studies [265]. We found that cGAS and STING were necessary for DNA induced DC maturation (*Manuscript I, Appendix I*)[212], supporting the evidence that cGAS and STING activation are particularly important for DC activation in response to DNA.

In *Study II* we used the direct STING agonist, cGAMP, and found that cGAMP could induce activation of DCs and macrophages (*Manuscript II, Appendix III*). cGAMP has the ability to slightly inhibit tumor growth when applied i.t. in murine tumor models [266]. However, the limited anti-tumor effects of pure cGAMP lead to an interest in finding approaches to enhance cGAMP delivery and function. We found that the depletion of cholesterol from cell membranes enhanced activation of DCs and macrophages in response to cGAMP, and that this was associated with enhanced activation of the STING pathway (*Manuscript II, Appendix III*). Low cholesterol levels are associated with increased membrane permeability, increased defense against viral infections, enhanced IFN-I production and increased attraction of T cells to inflammatory sites [252, 267, 268]. We observed that i.t. injection of cholesterol depletion reagent, MβCD, inhibited tumor growth in mice (*Manuscript II, Appendix III*). Both DNA and cGAMP can be released from tumor cells into the TME [269, 270]. Cholesterol depletion may aid cGAMP, and likely also DNA, to enter cells, including DCs. We also found that cholesterol depletion enhanced STING sequestering from the ER (*Manuscript II, Appendix III*). Collectively, these results show that cholesterol depletion increases the activation of the STING pathway in response to cGAMP and this promotes DCs to enhance adaptive immune responses.

The benefits of targeting and increasing the activity of the STING pathway to enhance anti-tumor responses is quite convincing. However, a few studies have observed some downsides of enhanced STING activity in tumor settings. In HPV-induced sarcomas, STING is found to be active in tumor cells, but in this type of cancer, activated STING

promotes IL-10, IDO and CCL22 induction and increases infiltration of Tregs, indicating promotion of a tolerogenic TME [271]. The induction of IDO associated with increased STING activity has also been observed in a Lewis lung carcinoma model, in which treatment with STING agonists induced tumor growth [272]. However, these features induced by activated STING was not observed in the B16 melanoma model [272]. These studies aid to the knowledge that activity of the STING pathway in cancer is very complex and might hold both beneficial and unbeneficial properties, dependent on the type of cancer. The downsides of overactivation of STING have also been established in autoimmune disease. SAVI (STING associated vasculopathy with onset in infancy), is an autoimmune disease, causing inflammation of the skin, blood vessels and lungs, and it is caused by a gain-of-function mutation of TMEM173, the gene encoding STING [273]. It is therefore important to balance the activity of the STING pathway, so that anti-tumor responses are promoted, and autoimmunity is avoided. Furthermore, it should be considered which types of cancer would benefit from treatment enhancing STING pathway activation.

Our studies suggest two different approaches to stimulate the STING pathway. Namely, by targeting a strong maturation stimulus, DNA, directly to DCs, or by enhancing the cellular response to cGAMP stimuli via a depletion of membrane cholesterol (*Manuscript I and II, Appendix I and III*) [212]. This increased activation of the STING pathway leads to enhanced IFN-I production, which has multiple beneficial impacts on immune responses against cancer. Since the immunological elimination of cancer contains multiple steps, as described in the cancer immunity cycle, targeting several steps in this cycle might enhance the efficacy of anti-tumor immunotherapy [9]. While checkpoint blockade therapy can help lift the restrain put on T cells in the TME, enhanced IFN-I production can increase T cell proliferation, their ability to induce tumor killing, and the generation of T memory cells [112-114]. Furthermore, IFN-I can enhance DC migration, maturation and cross presentation, and hence their ability to present TAs to T cells and induce T cell mediated tumor rejection [115-120]. The therapeutic approaches, described in *Study I and II* (*Manuscript I, Review I and Manuscript II, Appendix I-III*) [212, 213], targeting the STING pathway, which leads to DC maturation and enhanced IFN-I production, may therefore become a new addition to immunotherapy against cancer (**figure 6**).

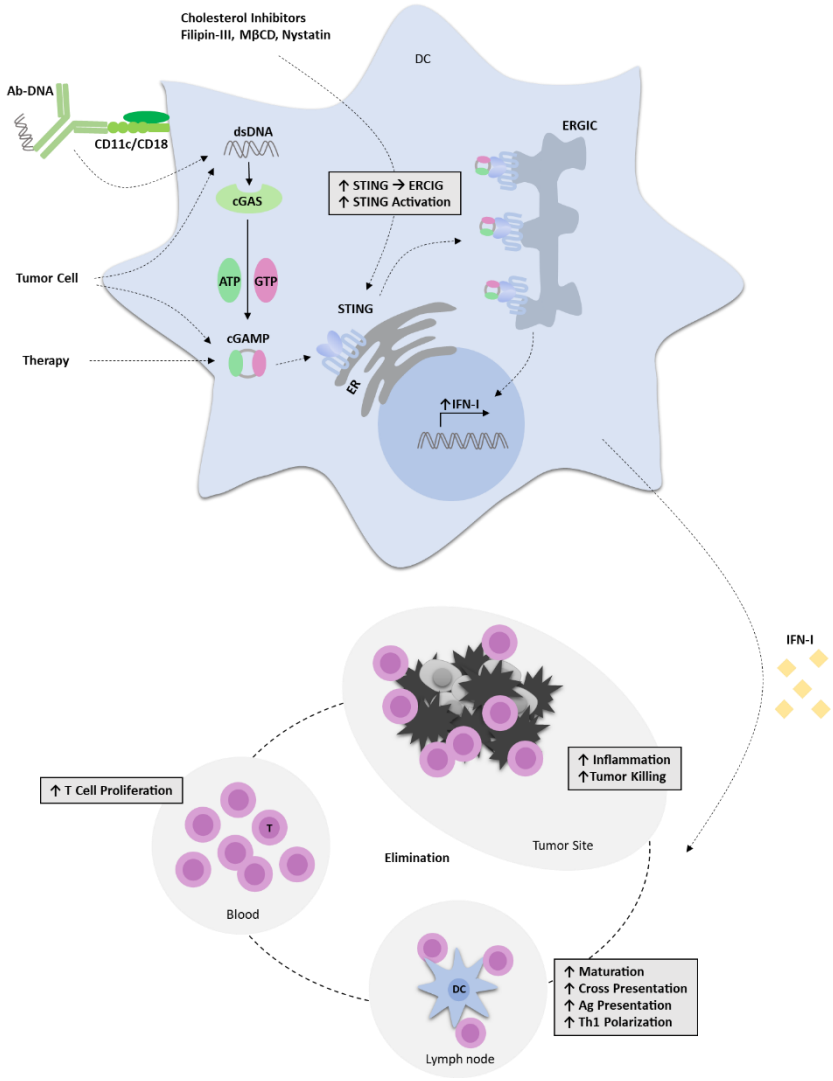

Figure 6. Activating the STING pathway as a strategy for anti-tumor immunotherapy. CD11c-targeted DNA and cGAMP can be delivered to DCs and induce activation of the STING pathway. DCs may also take in DNA and cGAMP released from tumor cells. Additional treatment with cholesterol inhibitors will further enhance STING activation, by enhancing STING release from the ER. STING activation leads to increased release of IFN-I, which increases DC maturation, cross presentation, and Ag presentation to T cells, and promotes Th1 differentiation. Furthermore, T cell proliferation is increased, and with combinational treatment with checkpoint inhibitors, this may lead to increased inflammation in the TME and enhanced tumor killing. Abbreviations: **cGAS:** cyclic GMP-AMP synthase. **cGAMP:** cyclic GMP-AMP. **STING:** Stimulator of Interferon Genes. **ER:** endoplasmic reticulum. **ERGIC:** ER Golgi Intermediate Compartment. **DC:** Dendritic cell. **T:** T cell.

CHAPTER 5. CONCLUSIONS AND FUTURE PERSPECTIVES

In the studies presented here, we found that the activation of DCs was possible by targeting DNA to CD11c and that this activation was dependent on STING and cGAS. cGAMP in combination with cholesterol depletion reagents is also able to activate immune cells in a STING-dependent manner, and the application of cholesterol inhibitors induce inhibition of tumor growth. These approaches, which activate the STING pathway, will therefore likely hold potential as applications for immunotherapy against cancer (*Manuscript I, Review I and Manuscript II, Appendix I-III*) [212, 213].

The STING pathway is highly important for IFN-I production and aids to link innate and adaptive immunity. It is becoming increasingly clear that the activity of the STING pathway can be manipulated via a variety of approaches, leading to formation of immune responses beneficial for anti-tumor immunity. However, the full complexity of this pathway still needs to be revealed. Studies mapping the pathway further, to gain a better understanding of how the activation and degradation of STING is regulated, will aid to the understanding of how it would be optimal to target the pathway with immunotherapy.

To prove that targeted DNA could be applied for cancer treatment, the Ab-DNA construct we generated in our study (*Manuscript I, Appendix I*) [212] should be tested in *in vivo* settings to see if this will be able to reduce tumor development and growth. Confirmation of the benefits of applying cholesterol depletion to reduce tumor growth should be made by applying a clinically relevant cholesterol inhibitor, e.g. Nystatin, as treatment in a tumor model. Applying the therapeutic strategies presented here may also be used in combination with other immune regulating treatment options for cancer, such as checkpoint blockade therapy, and it would be interesting to test if combinational treatment would enhance protection against tumors. Developing treatment options, which can decrease STING activity, may be useful for applications against autoimmune diseases. Investigation on how STING activity can be decreased could heighten our understanding of how manipulating the STING pathway affects the immune system in different diseases as well as how this can be utilized for development of new immunotherapeutic approaches.

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