Monocyte-derived dendritic cells in cancer immunotherapy

Considerations on their applicability

Dag Heiro Yi

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2021

UNIVERSITY OF BERGEN

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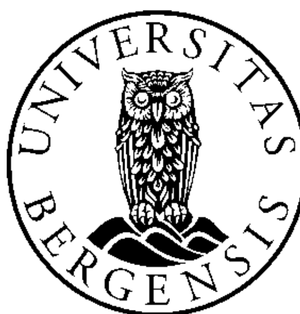
Scientific environment

The following doctoral work was conducted in the period 2012 to 2020, starting as a Medical Student of the Research Line, at the Broegelmann Research Laboratory (http://www.uib.no/en/rg/broegelmann), Department of Clinical Science, University of Bergen, within the framework of the Bergen Research School of Inflammation (http://www.uib.no/en/rs/brsi). The work was carried out under the supervision of Silke Appel and Roland Jonsson.



Acknowledgements

My journey to this Broegelmann started long before I even knew about Bergen. It was in elementary school that my homeroom teacher saw me sitting idly with nothing to do, and gave me a magazine she subscribed to, a magazine called Illustrert Vitenskap. I remember vividly reading about the concept of dendritic cells, these supposedly magnificent cells that could eradicate disease and potentially be the cure for cancer.

So, when I got the opportunity in 2012 to join the medical student research programme, two projects caught my eye. The first one was investigating the effect of Chinese medicine on cancer cell lines and the other was developing dendritic cells for immunotherapy. I applied for both and soon after, I was welcomed to meet **Roland Jonsson** and **Silke Appel** at Broegelmann research laboratory.

Looking back at it I remember one of the first things Silke remarked to me after our first meeting back in 2012, that surprisingly I wasn't confusing dendritic cells with neural cells. Meanwhile Roland gleefully remarked that he could add another flag to his office, disregarding the fact I'm born in Norway as mere details as I could speak fluent Chinese. The international environment is exactly what I was missing having been in Bergen. Colleagues were welcoming and discussions could take place without much worry, which was very important to me.

Silke for most parts is a paradox, stern but fair, understanding but blunt. It is hard for me to express how much gratitude I have towards you for your supervision and support during my time at the lab. It always amazes me at how many details you remember about things and the blistering pace you get things done. I highly doubt I would've gotten anywhere near where I am with the research without your guidance and supervision. Thanks for more or less being a motherly figure in all those years even though I've probably been a lazy kid for most parts.

Roland is like our lab grandpa. Always looking out for everyone and trying to make everyone succeed with their projects. Our discussions about Sweden have always amused me as your love for your home country is only rivalled by your love for an inclusive international work environment. Thank you for all those years of support.

Some of the first people I met were **Brith Bergum** and **Richard Davies**, who have helped me tremendously over the past 8 years with numerous difficulties. In fact, without your help, my hopeless chaotic nature would probably have been unable to move to my current apartment. Not just that, but this PhD dissertation would probably not have been possible without your help. So, thanks for helping me stay alive during those years.

Roman Volchenkov and **Florian Sprater** left not long after I started, but I still enjoyed Florian remarking about how he delivers my post and Romans love for miniature models.

The lovely technicians **Kjerstin Jakobsen** and **Marianne Eidsheim,** I have to thank you guys for helping me with many of my experiments and I always appreciate your willingness to help and answer my questions. In return I'll always respond quickly to help fixing your IT problems.

I also must express gratitude to **Kate Frøland** for helping me with all the administrative forms and bills. Without your guidance through the bureaucratic system I would probably still wonder whether travel expenses had to be paid privately or not.

Waqas Azeem, we collaborated quite early when you were in Silke's group, and again we collaborated for my final project. Thanks for all the discussions and cooperation in all those years. I also have to thank **Karl-Henning Kalland** and his group for fruitful collaborations and discussions. Much gratitude also has to go to **Magdalena Keindl**, thanks for helping me out countless times with questions on the flow panel and your insights on the matter.

Alexandra Petrovic, **Irene Sarkar** and **Anders K. Aarebrot**, while I met you guys a bit later during my period in the lab, I always enjoyed the discussions we had and especially the banter. Immunology might be the most prominent topic, but food was always right around the corner. Thanks for all the good times and laughs.

Tim D. Holmes, **Karl A. Brokstad** and **Veronica Binder**, I always enjoyed our discussions and musings about life. You guys have perspectives that are always interesting to hear and discuss, from Tim's worries about the Youtube comment section to Karls interest in getting old computer equipment from China.

Finally, I have to thank my family and friends outside the lab for supporting me all those years. Dad for supporting my venture into research, mom for always cheering me on, my sister for always being on Discord spamming me with random stuff and all my other friends who have put up with the strange organism that is me.

While my tenure as a PhD candidate might be over, who knows what the future will bring, at least I don't. If there's anything I've learnt those past years, it is that life is unpredictable, and so is research.

Abstract

Cancer is the leading cause of death in the developed world. While treatment options and detection have improved over the last century, mortality rate remains high especially in metastatic disease. Traditional treatment for metastatic cancer is often life prolonging with limited curative intentions. Furthermore, many traditional treatment regimens such as chemotherapy and radiotherapy have considerable side effects that drastically reduce quality of life. Prostate cancer is the second most common cancer in men worldwide and has limited treatment options with curative intention.

The introduction of checkpoint-inhibitors in treatment of melanoma showed that immune-response against cancer is possible, and that patients who respond have long survival.

Dendritic cell-based immunotherapy has shown to work in animal models and is a promising method to stimulate an immune response against cancer. One of the easiest methods to obtain dendritic cells (DC) is by generation from monocytes. These monocyte-derived DC (moDC) can be loaded with antigens and used to stimulate immune responses. However, recent clinical trials using moDC for immunotherapy showed disappointing results.

The overall goal of this thesis was to investigate factors that affect the properties of moDC such as culture conditions and maturation stimuli and how that affects their interaction with T-cells.

In paper I the effect of OK432 as a maturation stimulus for moDC was investigated in various formats. Properties such as phenotype, cytokine profile, migratory capacity and T-cell stimulatory capacity was measured. The addition of PGE_2 resolved the lack of migratory capacity in OK432 matured moDC. It was concluded that OK432 together with CL097 and PGE_2 is a promising cocktail for moDC maturation in immunotherapy.

In paper II the effects of the culture dish surface on the generation of moDC was investigated. Properties such as phenotype and cytokine profile were measured. Overall, the surface adhesion properties of cell culture dishes used significantly affected many properties of moDC in both immunogenic and tolerogenic culture conditions.

In paper III, moDC from patients with metastatic prostate cancer were investigated to determine if patient cells were capable of responding to maturation stimuli such as the

OK432 cocktail developed in paper I. MoDC from patients showed a mature phenotype and were able to stimulate autologous T-cells in an antigen-specific manner.

List of Publications & manuscripts

The doctoral thesis is based on the following publications.

I. **Yi, D.H**., Stetter, N., Jakobsen, K., Jonsson, R., Appel, S. (2018). 3-Day monocyte-derived cells stimulated with a combination of OK432, TLR7/8 ligand, and prostaglandin E_2 are a promising alternative for cancer immunotherapy, *Cancer Immunol Immunotherapy* 67**,** 1611–1620

II. Sauter, A., **Yi, D.H**., Li Y., Roersma, S., Appel, S. (2019). The Culture Dish Surface Influences the phenotype and Cytokine Production of Human Monocyte-Derived Dendritic Cells, *Frontiers in Immunology* 10: 2352.

III. **Dag H. Yi**, Waqas Azeem, Anne Margrete Øyan, Karl-Henning Kalland, Silke Appel. Effect of different maturation stimuli on phenotype and function of clinical grade dendritic cells from prostate cancer patients. *Manuscript*

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Abbreviations

AIDS	acquired immunodeficiency syndrome	moDC	monocyte derived dendritic cell
AP-1	activator protein 1	NACs	non-adherent cells
APC	antigen presenting cell	NK cell	natural killer cell
BCR	B-cell receptor	PAMP	pathogen associated molecular pattern
BCR-ABL	Breakpoint cluster region-Abelson tyrosine kinase oncogenic fusion gene	PBMC	peripheral blood mononuclear cell
CAF	cancer-associated fibroblast	PD-L1	programmed death ligand 1
CAR-T	Chimeric antigen receptor T	PD-L2	programmed death ligand 2
CCR7	CC-chemokine receptor 7	PMTs	photomultiplier tubes
CRISP/CAS9	clustered regulatory interspaced short palindromic repeat associated protein 9	Poly I:C	polyinosinic:polycytidylic acid
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester	PRR	pattern recognition receptor
CML	in chronic myelogenous leukemia	TAM	tumour associated macrophage
CTL	cytotoxic T-lymphocyte	TBK1	TANK-binding kinase 1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4	TCR	T-cell receptor
DAMP	damage associated molecular pattern	TGF-β	transforming growth factor beta
DC	dendritic cell	Th cells	T-helper cells
DNA	deoxyribonucleic acid	TIL	tumour infiltrating lymphocyte
ELISA	enzyme-linked immunosorbent assay	TLR	Toll-like receptor
FMO	Fluorescence Minus One	TMB	tumour mutational burden
GM-CSF	granulocyte-macrophage colony-stimulating factor	TNF- α	tumour necrosis factor α
HIV	human immunodeficiency virus	TNM	The TNM Classification of Malignant Tumors (T-tumor, N-lymph node, M-metastasis)
IFN	interferons	Treg	regulatory T-cell

IgG	immunoglobulin G	TRIF	TIR-domain-containing adapter-inducing interferon-β
IL	Interleukin	UICC	Union for International Cancer Contro
IRF3/7	interferon regulatory factor 3/7	V(D)J	Variable gene segments, Diversity gene segments and Joining gene segments
LPS	lipopolysaccharides	VEGF	vascular endothelial growth factor
MAPK	mitogen-activated protein kinase	WHO	World Health Organization
MHC I	major histocompatibility complex class I		
MHC II	major histocompatibility complex class II		
MLR	mixed leukocyte reaction		

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1. Introduction

1.1 The importance of the immune system

Since the dawn of life, the ecosystem is in a constant evolutionary race striving for survival through various methods. The main categories of life sustenance can broadly be categorized into primary producers, such as photosynthetic plants and subsequent consumers which involve herbivores and their predators. The most prevalent form of energy consumption among species is parasitism, siphoning energy from the organism's host. Without protection from parasitism, host organisms suffer the consequences of lower energy efficiency and usually lower chance of survival[1]. In the context of an infection with parasitical bacteria, viruses, fungi, protozoa or helminths, they are called pathogens, but this can also include malignant conditions which by extension can also be considered parasitical due to their energy draining features [2-4]. There are however also organisms that strive on symbiotic relationships with their host organism, which while surviving on the energy their hosts provide benefit the host organism overall through mechanisms such as aid in digestion[5]. While co-existing in this environment, it is in the best interest of living organisms to deter parasites and either encourage or ignore symbiotic relationships. To this end, larger multicellular-organisms have evolved a complex multi-layered system capable of distinguishing and rejecting harmful organisms from safe organisms called the immune system which is crucial for survival and many animal studies have shown that a disabled immune system often leads to early demise in a non-sterile environment or upon challenge with a pathogen[6, 7]. The human immune system is no exception. This leads to an evolutionary race between the parasitical pathogens and the human immune system where pathogens continuously evolve new ways to evade rejection from their hosts for their own survival. As the replication rate of most pathogens are considerably higher than that of humans, the mutational drive is in their favour. As congenital specific detection of each new mutation in the myriads of pathogens is impossible for the slow replicating larger host organisms, their immune system has evolved methods to effectively neutralize such threats with a combination of a

germline preserved innate immune system for broad general protection, and an adaptive immune system hypermutating to specialize against specific pathogens[8].

1.1.1 The innate immune system

Often regarded as the first line of defence, the innate immune system is the evolutionary preserved part and is especially important early in life. One of the most obvious defences against pathogens are physical barriers such as our skin. Not only does it act as a physical barrier, but skin cells also secrete anti-microbial peptides such as defensins and cathelicidins which are capable of neutralizing many different pathogens[9]. The mucosa lining the gastrointestinal tract, the respiratory tract and the urogenital tract also contain both physical and chemical properties to deter pathogenic infection[10, 11]. Past the outer barriers, the liver also secretes immunogenic proteins as part of the complement system which is capable of neutralizing and coat pathogens through a process called opsonization. This not only has the potential of neutralizing pathogens, but also makes opsonized pathogens easier to detect for the rest of the immune system. Apart from barriers of the innate immune system, there are many different specialized cells with unique roles that comprise the active part of the innate immune system consisting of macrophages, neutrophils, eosinophils, natural killer cells (NK cells), monocyte, dendritic cells (DCs) and others as shown in figure 1 [12]. Lately a new class of innate immune cells were classified into three different groups in 2013 by Spits at al named innate lymphoid cells that appears to be important in mucosal immunity but are not shown in the figure [13]. How innate immune cells function, communicate with other cells and react to their environment is dictated by chemical signalling molecules of the immune system called cytokines such as interferons (IFN), interleukins (IL), and chemokines as well as surface proteins such as adhesion molecules affects the tissue localization of immune cells and how they bind and interact with other cells [14].

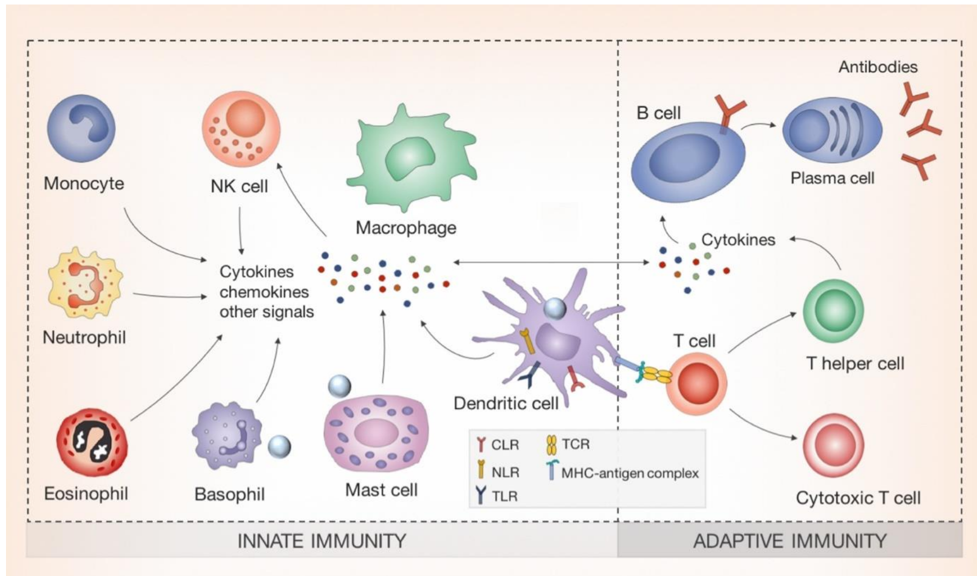

Figure 1. *Overview of the central role dendritic cells play in the immune system. Upon activation of dendritic cells through antigen binding to pattern-recognition receptors, including C-type lectin receptors, leucine-rich repeat-containing receptors and Toll-like receptors, dendritic cells will secrete cytokines that activate natural killer cells and modulate T cell differentiation. Recognition of antigen-Major Histocompatibility Complex on dendritic cells by the T Cell Receptor will also impact on T cells expansion. Figure adapted from Silva et al [15]. Reprinted and modified with permission from Elsevier*

1.1.2 Receptors and signalling pathways of the innate immune system

While pathogens are continuously replicating and mutating, most of them have evolutionary conserved regions that the cells of the innate immune system have evolved receptors for. In fact, immune cells have myriads of different receptors targeting various pathogenic stimuli. These pattern recognition receptors (PRRs) can recognize components directly from pathogens called pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) which are absent in all human cells and highly associated with pathogenic gram-negative bacteria[16, 17]. One of the

most researched PRRs belong to the group called Toll-like receptors (TLR) which humans have 10 of and comprises of 6 surface receptors and 4 intracellular receptors as shown in figure 2 [18].

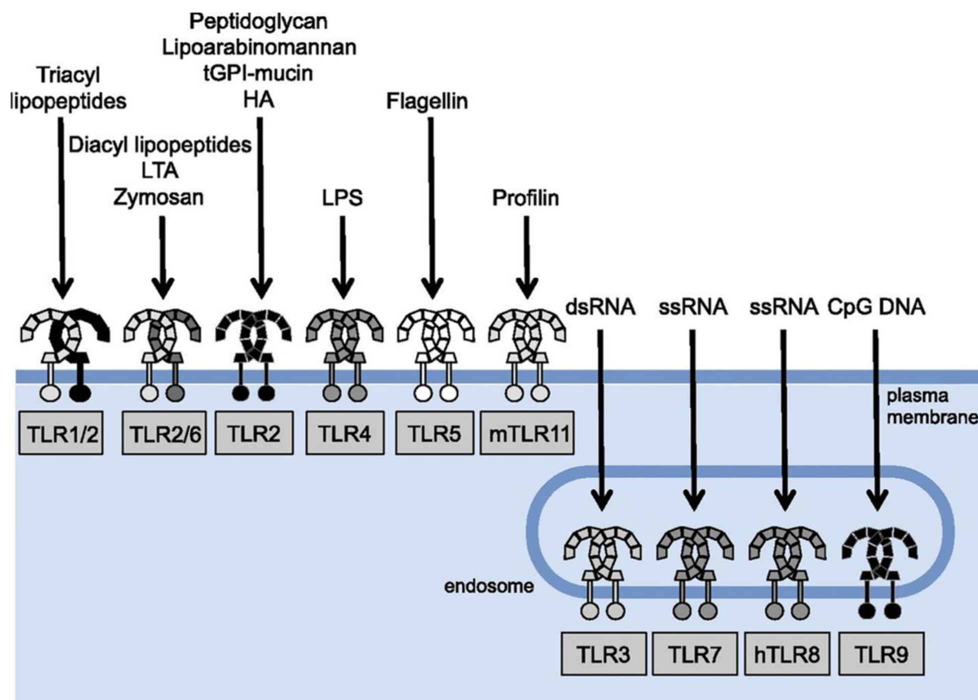

Figure 2. *Overview of Toll-like receptors found in human and mouse, main ligands and cellular localization (hTLR and mTLR denote Toll-like receptor retricted to human and mouse respectively). Figure republished with permission from El-Zayat et al* [18].

Each Toll like receptor triggers an intracellular downstream signalling cascade upon binding to its respective PAMP ligand, usually resulting in the activation of NFκB through the MyD88 pathway or TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway. A simplified overview of the main TLR activated signalling cascades is presented in figure 3.

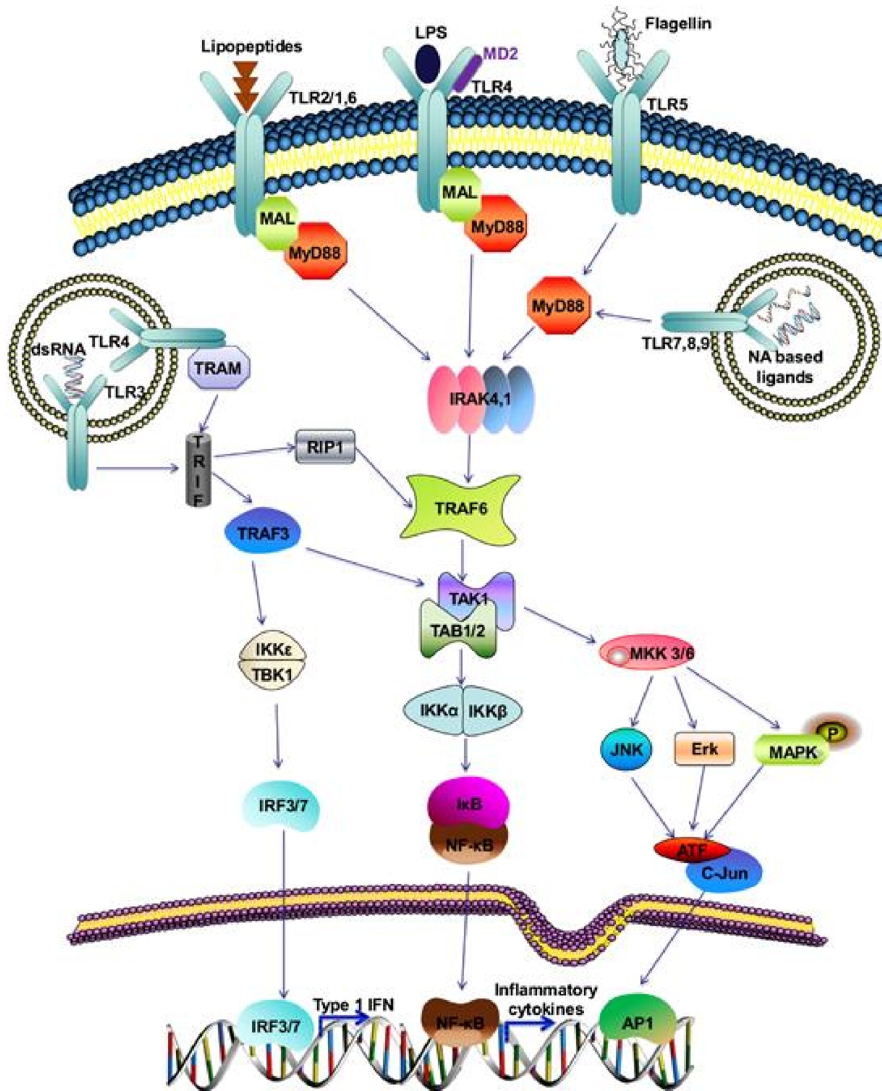

Figure 3. *Intracellular signalling pathways involved in cellular responses to Toll-like receptor activation. Following TLR binding to its corresponding ligand information is transferred through a series of steps ultimately resulting in the transcription of genes modifying cellular responses. TLR activation commonly leads to the production of Type I IFN though the activation of interferon regulatory factor (IRF) and inflammatory cytokines through NFκB and AP1. Figure by Anwar et al.* [19]

NFκB is a key regulator of many genes involved in inflammation and controls among others the production of pro-inflammatory cytokines in immune cells. TRIF activation can also lead to activation of TANK-binding kinase 1 (TBK1), subsequently ending in production of type 1 interferons through translocation of phosphorylated interferon regulatory factor 3/7 (IRF3/7) into the cell nucleus [20]. An additional branch for both pathways mentioned above is through activation of mitogen-activated protein kinase (MAPK) resulting in activation of activator protein 1 (AP-1) which regulates genes affecting proliferation[21]. The immune cells have also evolved receptors to detect signals of cellular stress and destruction, such as free deoxyribonucleic acid (DNA), organelle components or other cell particles normally not in the extracellular matrix, called damage associated molecular patterns (DAMPs). These receptors are crucial for immune cells to detect pathogens and cellular damage in the surrounding tissue and highly dictate their subsequent response to such stimuli [17, 22].

1.1.3 Immune cells of the innate immune system

Usually the first cells to encounter infiltrating pathogens, macrophages are immune cells residing in tissues and received its name due to its size and strong ability to “eat” particles in a process called phagocytosis which is one of the methods used by immune cells to neutralize pathogens. Apart from their role in combating pathogens, they play a key role in regulating the response of the immune system as well as regulating tissue repair after local damage. Cross-talk between macrophages and other immune cells is vital in determining how the immune system reacts towards pathogens as they have the ability to promote inflammation by secreting proinflammatory cytokines such as tumour necrosis factor α (TNF-α) and recruiting other immune cells, notably neutrophils through chemokines such as IL-8, to neutralize potential pathogens causing the inflammation. In this inflammatory state, they are often called M1 macrophages. However, they also play a key role in tissue repair and immunosuppression in the absence of pathogenic stimuli which is often referred to as M2 macrophages. Macrophages are alongside DCs considered antigen presenting cells (APCs) because of their ability to process and present peptide antigens on major histocompatibility complex class II (MHC II) [23-26].

Neutrophils are the most numerous immune cells usually numbering over half the total amount of white blood cells in humans. They are blood circulating immune cells that respond to inflammation and recruited through localized vasodilation caused by tissue residing cells and subsequent migration into target tissue. As the most abundant innate immune cell, they have strong anti-pathogenic capabilities through phagocytosis, degranulation resulting in release of degrading enzymes and creation of extracellular traps formed by DNA fibers and proteins [27].

Monocytes are large blood circulating immune cells with the capability of developing into macrophages. They are able to migrate into tissue to replenish local macrophage numbers and under certain circumstances develop into DCs. While monocytes do have antigen presenting capabilities similar to their post-differentiated specialized versions, whether this has a function in their undifferentiated form in vivo remains unclear [25, 28].

Eosinophils, basophils and mast cells are important cells for their anti-helminthic responses being able to stimulate and coordinate physiological and immunological reactions against large pathogens otherwise highly resistant to other immune cells [29-31].

NK cells are often regarded as the innate immune system's anti-viral specialists as they can neutralize infected host cells. They are considered the primary immune cells capable of eliminating cells not expressing major histocompatibility complex class I (MHC I) through probing cell surfaces with various MHC I binding receptors. This mechanism usually acts as a safeguard against pathogens or malignancies that attempt to hide from the immune system through manipulating antigen presenting functions [32].

Dendritic cells are one of the most recently discovered immune cells by Steinman and Cohn in 1973 and are often described both as sentinel cells and as professional APCs [33]. This is because unlike macrophages who primarily affect and regulate local tissue site, the primary role of DCs is to present antigens to other immune cells and communicate the context of the antigen presented. Acting as the scouts of the immune system, DCs, as their name suggests, have long probing dendrites to be able to gather

antigens from most of the human body. Upon uptake of antigen together with stimulation, DCs undergo a maturation process which enhances their ability to present antigens by upregulating expression of MHC class II and costimulatory molecules CD80 and CD86 as well as upregulate their expression of CC-chemokine receptor 7 (CCR7) which stimulates chemotaxis towards lymph nodes to meet näive T-cells. A commonly used marker for mature DC is CD83 as it is clearly upregulated in mature DCs, but its function is as of yet not very clear [34]. Those antigen presenting properties and their ability to activate cells of the adaptive immune system are why DCs are considered the link between the innate and the adaptive immune system [35, 36].

1.1.4 Limitations of the innate immune system

As the evolutionary conserved part of the immune system, innate immune cells are by nature limited in their specificity as their PRRs are germline encoded. While this can protect against most pathogenic species, there are many exceptions. As pathogens replicate and mutate, strains develop that have properties that helps avoiding the immune system. This could be by hiding or weakly express conserved regions to avoid strong PRR binding, changing properties of capsule or membrane to resist detection or neutralization, manipulating immune cell signalling pathways, structure mimicry to host antigens, release of decoy superantigens and by various other means as shown in figure 4. While some cells of the innate immune system have shown memory function and degrees of adaptability, it is not enough to overcome all the immune evasive strategies pathogens evolve. To cover the insufficiencies of the innate immune system, slower replicating organisms evolved the adaptive immune system to overcome those constraints and widely believed to have originated from Gnathostomes hundreds of millions of years ago [2, 37-42].

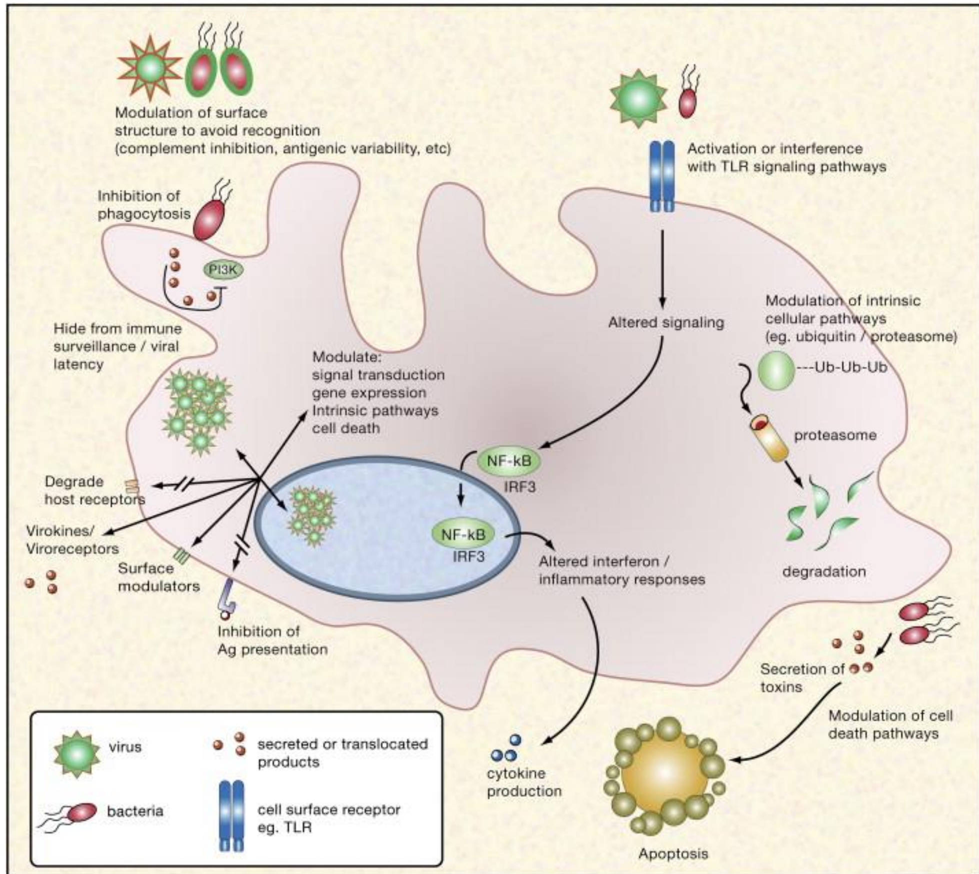

Figure 4. *Immune evasion strategies of viral and bacterial pathogens. Viral and bacterial pathogens employ a number of strategies to avoid mediators of an immune response and their subsequent destruction. These strategies range from inhibition of antigen presentation and inflammatory responses, inhibition of phagocytosis and modulation and degradation of cellular receptors. Figure from Finlay and McFadden 2006 [40]. Reprinted with permission from Elsevier.*

1.2 The adaptive immune system

As pathogens continuously evolve and acquire new structural changes that can interfere with detection by our germline inherited PRRs, it is unfeasible for every immune cell to be capable of reacting against every possible genetic rearrangement of these new pathogenic strains or pre-emptively have enough quantity of diverse immune cells to cover every possible new strain. To solve this conundrum, the adaptive immune system has evolved with a reactive strategy that maintains very high variability in what it can react against that only gets expanded when required in a process called clonal expansion. In comparison to the innate immune system, the adaptive immune system requires time to adapt to new challenges but offers a much more robust and specific immune response [8].

1.2.1 The development and cells of the adaptive immune system

The adaptive immune system mainly consists of T-cells and B-cells and their subtypes and the humoral immunity. Compared to the innate immune system, the adaptive immune system has a much wider repertoire of possible antigens it can recognize.

The way which the adaptive immune system can cover such a diverse repertoire of potential receptors for new antigens is due to somatic recombination of Variable gene segments, Diversity gene segments and Joining gene segments (V(D)J). These gene segments are responsible for coding the binding site of adaptive immune cell receptors called B-cell receptors (BCR) and T-cell receptors (TCR) for B and T cells, respectively. Due to the highly diverse ways which the V(D)J segments can be rearranged and recombined, the resulting TCRs and BCRs are able to bind to novel antigens that have never been encountered before [43, 44].

As mentioned previously, due to V(D)J recombination during the development of progenitor adaptive immune cells, peptides expressed in our own body are not excluded from this coverage. As mounting an immune reaction towards the host’s own cells is highly detrimental, it is crucial to neutralize immune cells which can bind self-peptides in an immunogenic way, known as autoreactive immune cells. The process of neutralizing autoreactive cells is called negative selection which leads to the concept

of self-exclusion by the immune system called central tolerance. Similarly, immune cells which do not have the capability of mounting a strong immune response are of limited use to the immune system and are therefore also removed. To undergo these processes, a specialized organ prominent in childhood called the thymus is where progenitor cells undergo maturation by receiving a survival stimuli if they bind strongly to MHC molecules and a signal to undergo programmed cell death (apoptosis) or go into anergy if they bind strongly to self-peptides presented by medullary thymic epithelial cells. Some of the self-reactive immune cells can also turn into protective regulatory cells that inhibits immune reactions towards their antigen. A similar process also happens with B-cell progenitors in the bone marrow where positive and negative selection also occur [45-48].

The key to an adaptive immune response lies with how it interacts with MHC molecules which are one of the main triggers activating the adaptive immune cells. As previously mentioned, the immune system keeps a vast repertoire of immune cells capable of reacting towards various peptides, but without having their ligand presented to them, they remain circulating and inactive. These cells are called näive immune cells and they circulate between peripheral lymph nodes. [49, 50]. As the primary presenter of antigens from peripheral tissue to immune cells residing in the lymph node, DCs are the primary activators of an adaptive immune response in T-cells. How DCs present the antigen is crucial in determining what type of response the naïve T-cells will have and this can generally be split into 3 key signals as shown in figure 5, the first being the peptide presented on MHC binding to the TCR, the second being co-stimulatory or inhibitory molecules on the surface of the cells binding to their ligands and the third being the secreted cytokines from the DCs [51]. If all 3 signals are pro-inflammatory, bound naïve T-cells are selected to undergo a process called clonal expansion where T-cells with the strongest binding to the antigen presented undergoes extensive division resulting in large amounts of clonal daughter cells [52, 53]. Naïve B-cells also undergo a similar process where strong attachment to presented antigens in an inflammatory context leads to clonal expansion [54].

The 3 signals determine which subset of adaptive immune cells gets clonally expanded and influences their function. In the context of a peptide presented in the context of high co-stimulatory signals from CD80 and CD86 on the surface of DCs together with secreted proinflammatory cytokines such as IL-12p70, naïve T-cells can develop into effector CD4+ T-helper cells (Th cells) or CD8+ cytotoxic T-lymphocytes (CTLs). Conversely if the signals skew towards inhibitory signals such as expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on T-cells or programmed death ligand 1 (PD-L1) and 2 (PD-L2) on DC, which are part of signal 2 together with secretion of regulatory cytokines such as transforming growth factor beta (TGF-β) as part of signal 3, leads to no reaction or expansion of regulatory T cells (Tregs) that suppresses immune reactions [55-57].

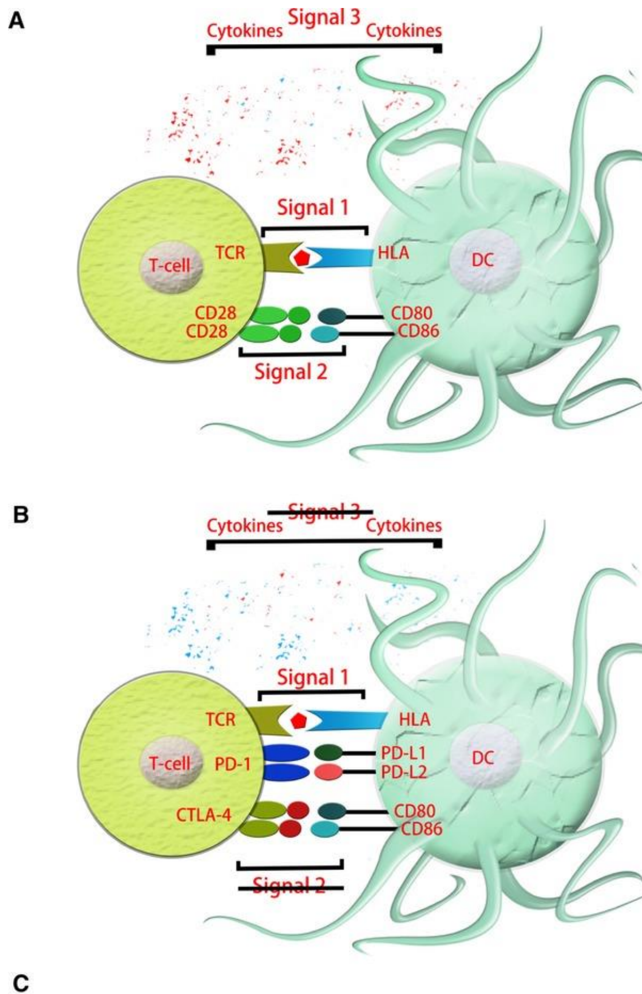

***Figure 5**. The three signals of antigen presentation. T cell activation requires three signals from APCs such as DCs to become activated or primed. First, T cells must recognise their cognate antigen in context with HLA. Second, T cells require a costimulatory signal (CD80/86) from the APC. Finally, cytokines from immune cells and the microenvironment determine the T cell phenotypic fate. Conversely APCs can inhibit T cell responses through presentation and binding of programmed death ligand 1 (PD-L1) and 2 (PD-L2) with T-lymphocyte-associated protein 4 (CTLA-4) together with regulatory cytokines. Figure from Yi and Appel 2013* [51] *republished with permission from* John *Wiley & Sons Ltd*

1.2.2 The functions of the adaptive immune system

Due to the selective nature of how effector cells of the adaptive immune system develops as described in the previous section, the adaptive immune response is much more specific than the innate immune system and can thus mount a much more robust immune response with very small risk of autoreactivity. The immune response can roughly be split into two categories, namely cellular immunity and antibody mediated immunity also known as humoral immunity [8, 58, 59].

T-cells are the cells responsible for cellular immunity and different subsets carry different roles. The 3 main subtypes of T-cells are CD4+ Th-cells, CD8+ CTLs and Tregs. Th-cells mediate immune responses by secreting cytokines and providing stimuli to other immune cells, notably macrophages and B-cells, to mount a much stronger immune reaction than they would otherwise. This results in more rapid phagocytosis and elimination of pathogens expressing the peptide binding to the Th-cells. They are also vital in regulating the response of other immune cells such as CTLs and NK cells through cross talk [41, 60-62]. The response they induce is highly dependent on the phenotype as well as the cytokine profile of these cells such as expression of CD40L on the surface and secretion of IL-6 and IFNγ [63, 64].

CTLs on the other hand take a more direct approach in eliminating pathogens by storing and releasing granules containing perforins and granzymes upon being presented their TCR specific peptide by MHC on target cells [65]. They are especially adept at eliminating targets that hide within other cells and therefore resistant to phagocytosis such as viruses and are also vital in eliminating own cells that express PAMPs associated with malignancy [66, 67].

A prolonged immune response can be highly detrimental as chronic inflammation is related to various ailments and can often result in diseases or be part of a group of diseases called autoinflammatory diseases if no autoantigen is known [68]. Therefore, it is important to not prolong immunogenic responses more than necessary and to tolerate harmless or symbiotic organisms such as many gut bacteria. If the immune system is unable to completely neutralize a pathogen after prolonged immune activity but detects limited further pathological activity from its target, in many cases it instead

learns to tolerate it. Tregs are the key regulatory cells responsible for suppressing unwanted immune responses against such targets and are prevalent in tissue close to symbiotic or harmless organisms. They do so by secreting immunosuppressive cytokines such as IL-10 and TGF-β as well as expressing inhibitory surface molecules such as CTLA-4 [69-71].

The humoral immunity provided by B-cells consists of antibodies which are secreted BCRs. Depending on the type of stimuli they receive during the maturation process, the receptor undergoes isotype switching to fit the context in which the target chemical molecule is presented. Similarly to the proteins of complement system of the innate immune system, but in a much more specific manner, antibodies coat and opsonize their targets both to label them as targets for other immune cells as well as neutralizing the functions of the target [8, 72, 73].

A shared trait among adaptive immune cells is the need for continuous survival stimuli. This is in the form of cytokines such as IL-2, a key survival signal, which is auto secreted by T-cells upon binding antigen to their respective TCR. In B-cells, several cytokines such as IL-21 and IL-4 are considered survival signals and are mainly secreted by Th-cells residing in the follicles of lymph nodes [74-76]. However, once the target peptide is eliminated from the body and survival stimuli is no longer provided, most of the effector cells of the adaptive immune system undergo apoptosis as they no longer are needed. However, a subpopulation of long surviving dedicated memory cells of both T and B lineage remains post-clearance of antigen in anticipation of a reoccurrence of their target antigen and are ready to undergo clonal expansion upon re-detection of target antigen. This way the adaptive immune response bypasses the time needed during novel naïve activation of adaptive immunity as well as immediately clonally expand antigen specific T-cells which is why establishment of memory is the core objective of vaccination [77-79].

1.2.3 The vulnerabilities of the adaptive immune system

While the adaptive immune system is extremely powerful and versatile, it is not without its flaws. There is a possibility for central tolerance to fail, and autoreactive immune cells escape neutralization which can lead to autoimmune diseases such as Myasthenia Gravis and Systemic *lupus* erythematosus [48, 80, 81]. Even if the adaptive immune system targets a pathogen associated antigen, the response needs to be appropriate for the purpose of eliminating the threat. If the immune response is not robust enough, the pathogen might escape elimination resulting in continuation of the disease, but conversely if the immune reaction is too strong, it can lead to a breakdown of immune regulation and cause a deadly physiological condition called a cytokine storm [82, 83].

There are also ways how pathogens can evade even the adaptive immune system by similar mechanism to how pathogens evade the innate immune system such as mimicry, diversion with superantigens or even directly inactivating components of it. A well-known pathogen that is capable of deactivating the adaptive immune response is human immunodeficiency virus (HIV) which infects CD4+ Th-cells resulting in their eventual destruction and leading to acquired immunodeficiency syndrome (AIDS) [84, 85].

The ability to undergo clonal expansion can also be a double-edged sword in some circumstances if the regulatory mechanisms such as requirement of survival stimuli fail, a rapid uncontrolled expansion of immune cells leads to a group of cancers called lymphomas and leukemias [86, 87].

Overall, the innate and adaptive immune systems complement each other by covering many of each other's weaknesses, namely the innate immune system covering for the slow initial phase of the adaptive immune response and the adaptive immune system covering for the innate immune systems limited repertoire of receptor specificity. Together this makes for a very powerful biological system that has protected us since the dawn of humankind.

1.3 Cancer

Humans are living longer than they have ever lived before. Myriads of factors contributing to this are among others the advance of modern medicine and better coverage for necessities such as clean water, food security and housing has drastically improved life expectancy all over the world. The introduction of vaccines has drastically reduced death by previously common diseases such as smallpox and polio [88, 89]. In fact the rapid rise of average life expectancy all over the world since the past century is lauded as one of the greatest accomplishments in human history and is still projected to continue rising in the future [90, 91]. General better health awareness and better access to a diverse diet has also reduced the impact of lifestyle related mortality factors such as smoking and cardiovascular diseases in high income countries [92-94]. As cancer is a disease with incidence risk increasing by age, all of those factors has led to cancer becoming the most common cause of death in developed countries [95-97].

Cancer is one of the leading causes of death worldwide with the World Health Organization (WHO) reporting 9.6 million casualties in 2018 and accounts for almost double the amount of deaths compared to cardiovascular diseases in developed countries [95, 98]. Cancer is a very broad group of different diseases with different mortalities, progression and symptoms but with certain common traits. In one of the most influential papers published about cancer in 2011 titled "Hallmarks of Cancer: The Next Generation", Hanahan and Weinberg outline eight biological conditions that are hallmark features of cancer [4]. These consist of evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumour-promoting inflammation, activation of invasiveness and metastasis, inducing angiogenesis, genome instability and mutation, resisting signals of cell death, deregulation of cellular energetics and sustaining proliferative signalling as shown in figure 6. This modern interpretation of cancer activity has paved the way for more targeted therapies against these hallmarks [99-103]. However, many such targeted therapies are only recent discoveries and are still under research, development and optimization.

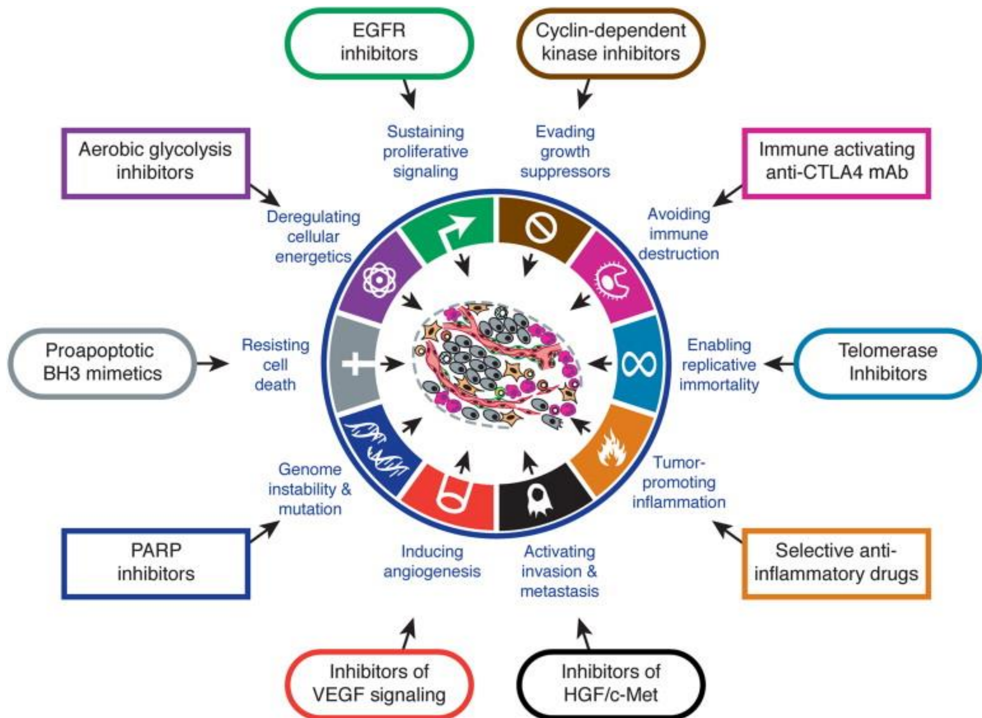

Figure 6. *The hallmarks of cancers and potential targets for therapy as presented by Hanahan and Weinberg 2013. Cancers acquire functional capabilities that allow cancer cells to survive and proliferate (inner ring). Drugs which interfere with these capabilities have been developed for their potential use as cancer therapeutics. Figure from Hanahan and Weinberg 2013* [4]. *Republished with permission from Elsevier.*

1.3.1 Cancer as a genetic disease

Unlike pathogens such as bacteria and viruses, cancer is by its nature a disease caused by genetic modification of our own cells leading to uncontrolled growth. Every time a cell undergoes mitosis, its DNA must be pulled apart and replicated. Each time this happens, there is an inherent risk of spot mutations or minor changes to the genome during the replication stage. Mutations can also be instigated by viruses that alter the genome for their own survival and replication such as Epstein-Barr virus and Human Papilloma virus. Spot mutations are necessary for evolution of the species as outlined

beautifully in the concept by Darwin in his revolutionary work “On the Origin of Species” where mutations positively affecting a cells contribution to the survival of its multicellular system gets its genome inherited by its descendants [104]. By the same system, there is a risk of mutations hitting key growth regulating genes resulting in uncontrollable growth or mutations rendering the cell unviable. To prevent this from occurring, our cells have many checks and balances involving various enzymes that scan and repair genetic errors during mitosis. Our genome also encompasses sequences coding for proteins acting as regulators of growth and genetic stability[105].

One of the most studied and most commonly occurring mutation resulting in cancer are mutations in sections of the tumour protein p53 gene, a transcription factor comprising of five regions with high degree of conservation crucial in maintaining genetic stability and regarded as one of the most important tumour suppressor genes [106]. Ever since its discovery in 1979, its role in cancer has been extensively studied and many experiments have shown that it is vital for cell-cycle arrest, induction of senescence or induction of apoptosis in cells before they develop into cancers. Knockout experiments of p53 in mice have shown that these mice have a dramatically reduced lifespan solely due to development of early tumours cementing the importance of tumour suppressor genes in preventing cancer [107-109].

While tumour suppressor genes are important in the prevention of cancer, there are also genes that are over-expressed or mutated in certain types of cancers that contribute to their growth and survival referred to as oncogenes. Prominent examples include HER2 and BRCA1/2 in breast cancer [110, 111] and BCR-ABL in chronic myelogenous leukemia (CML) [112, 113].

Further evidence of cancer being a genetic disease can be observed epidemiologically by assessing environmental risk factors. A key driver for mutations is oxidative stress and tissue conditions that promote this such as chronic inflammation or lifestyle habits such as smoking clearly increases the risk of developing cancer [114-117].

1.3.2 Immune evasion in cancer

As previously mentioned, most potential cancers are stopped at conception by checks and balance mechanisms during DNA replication. However, even if tumour suppressor genes fail to stop the development of cancer, there is emerging evidence for the role of immune surveillance, the hypothesis that many pre-cancerous cells are eliminated by the immune system before they can develop into full blown cancer by recognizing onco-antigens as non-self. Several animal experiments show rejection of transplantation of tumour tissue in syngeneic animals, but tolerance of healthy tissue. This alongside detection of tumour infiltrating lymphocytes (TILs) in the grafts upon rejection provides evidence that the immune system is capable of detecting onco-antigens and react against cancer [118, 119]. While tumour graft experiments cannot be conducted on human twins for ethical reasons, TILs are highly associated with better prognosis in many types of cancers [120-122]. Many of the discoveries in animal models regarding immune surveillance, however, have been hard to translate into human settings as concepts such as IFNγ and perforin treatment that appear to contribute to anti-tumour immune responses in mice were not observed in human patients of various cancer types [123].

The prevalent model for immune outcomes regarding cancer is the concept of the three E's, namely Elimination, Equilibrium and Escape [124]. As discussed previously, there is ample evidence that the immune system is indeed able to eliminate cancers. However, cancers are not homogenic diseases, and unless the immune system manages to eliminate all cancer cells, it might instead promote Darwinian selection of the cells that escape elimination. Equilibrium is the proposed state in which immune cells continue to eliminate and control the growth of detectable tumour cells, but at the same time driving a selective survival of immune resistant tumour cell strains. The final phase of this model results in immunological escape, where detectable tumour strains have mostly been eliminated and now the tumour consists primarily of immune resistant strains which growth can no longer be controlled as shown in figure 7 [119, 124].

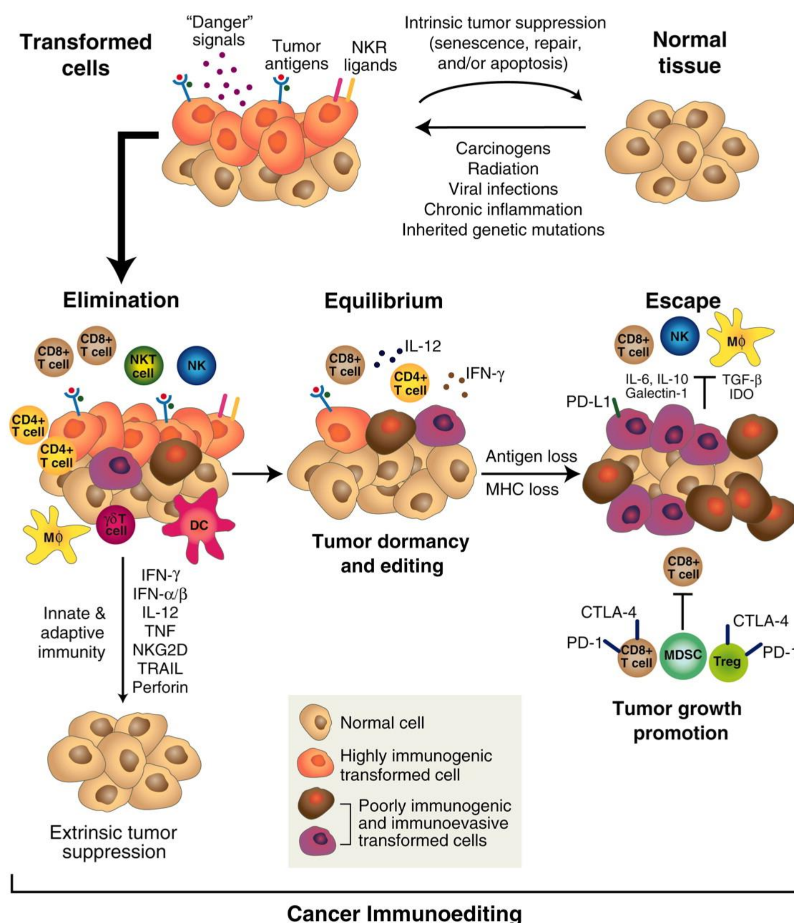

Figure 7. *The three E's of cancer immunity (Elimination, Equilibrium and Escape). The figure illustrates the prevalent model for immune outcomes regarding cancer. In the elimination phase, innate and the adaptative systems act to destroy developing tumors. If unsuccessful and a rare cancer cell variant survives, it may enter the equilibrium phase, where outgrowth is prevented by actions of components of the immune system in particular T cells, NK cells, IL-12 and IFNγ. An outcome of selective pressure on tumor cells may result in tumor cells that are able to escape the regulation by the immune system. These tumor cells enter the escape phase and cause clinically apparent disease. Figure from Schreiber et al 2011. Reprinted with permission from American Association for the Advancement of Science.*

Due to the nature of cancer originating as a genetic defect of our own cells, they are inherently different from pathogens who have clear components or molecular patterns that humans do not have. While some cancers have prominent antigens that do not normally exist in our body such as BCR-ABL fusion protein in CML, the very existence of CML to begin with shows that just expression of an aberrant protein is not always enough to elicit a robust immune response [125]. To further use CML as an example, despite BCR-ABL fusion being a requirement for CML diagnosis and clearly related to malignancy, there are reports that a small amount of healthy individuals have this mutation in the normal population, suggesting that immune cells that recognize this fusion protein are indeed either absent or inactive in patients [126]. Evidence that the immune system can be activated to react against BCR-ABL was shown in recent experiments showing promising result in BCR-ABL vaccination capable of stimulating an immune response against BCR-ABL expressing cells [127, 128].

The difficulty in detecting clear PAMPs in often DAMPs rich cancer microenvironment can lead to further immune evasion through the function of regulatory cells. As mentioned previously, the phenotype of innate immune cells depends on their environmental context through binding on PRRs. As many tumour cells do not express clear PAMPs that are detectable in a pathogenic context by innate immune cells, the main stimuli they receive are that of DAMPs due to destructive tumour activity, and there are numerous studies that show these tumour associated macrophages (TAMs) as promoters of tumour growth, angiogenesis and drivers of metastasis [129-132]. The metabolic rate of cancers also leads to a hypoxic microenvironment as well as induce pH changes that further promotes angiogenesis due to secretion of Vascular Endothelial Growth Factor (VEGF) by pericytes as a response [133].

Cancer cells are also able to convert local fibroblasts into cancer-associated fibroblasts (CAFs) through various stimuli such as cytokine stimulation and alternation of their extracellular matrix. This in turn aids cancer in both growth and immune evasion by hiding in a capsule of immunosuppressive non-cancerous cells [134, 135].

Furthermore, there is ample amounts of evidence showing that many types of cancer attract Tregs through chemokines induced by the hypoxic microenvironment such as CCL12, CCL22 and CCL28 or directly secreted by the tumour cells themselves which leads to local immunosuppression through secretion of regulatory cytokines or expression of inhibitory surface molecules [136-138].

Additionally, it has been shown that some types of cancer such as malignant melanomas and non-small celled lung cancer directly inhibit effector immune cells by expressing inhibitory molecules such as PD-L1 [139, 140].

1.3.3 Classical therapies for cancer and limitations

Treatment for cancer has always been highly affected by the timing of diagnosis. By far the most successful and oldest method of therapy has been surgical resection with wide margins or complete removal of the affected organ if possible. The globally recognized standard of charting the progress of cancer is through the Tumour- Node-Metastatsis Classification of Malignant Tumors (TNM) developed by the Union for International Cancer Control (UICC) (Figure 8). While different cancers have different criteria for their TNM staging, the core concept remains the same.

Classification	Definition
Tumor	
Tx	Tumor cannot be evaluated
T0	No evidence of a primary tumor
T1-4 a-b-c	Refers to size of tumor Letters refer to specifics of different cancers
Nearby lymph nodes	
Nx	Nearby lymph nodes are not evaluated
N0	No cancer cells are found in nearby lymph nodes
N1, N2, N3 etc	Cancer cells are found in the written amount of nearby lymph nodes
Distant metastasis	
MX	Metastasis cannot be measured
M0	Cancer has not spread to other parts of the body
M1	Cancer has spread to written amount of other parts of the body

Figure 8. *General criteria for the different TNM staging parameters.*
The figure illustrated how to interpret the three different main parameters of TNM staging classification as established by UICC. Figure created based on UICC guidelines 8th edition [141]. Created with permission from John Wiley and Sons Ltd.

While surgery is highly relevant in early stages of cancer and prognosis is usually good as long as the tumour has low TNM staging, once metastasis occurs the prognosis usually drops drastically, but to what degree depends on the cancer type and can be disputed depending on how cause of death is classified [142-144].]

The traditional methods of treating metastatic cancer are by chemotherapy, which is an umbrella term for treatment with various drugs aimed at inhibiting growth processes such as mitosis. As uncontrolled growth is one of the hallmarks of cancers, inhibitors of mitosis were some of the first chemotherapy drugs used. This led to the development of anti-folates which inhibit de-novo DNA synthesis even though the mechanisms of action were unknown at that time [145]. Today the anti-folic drug methotrexate introduced in 1950 is one of the most widely used drugs in treatment of cancer and other conditions where reduction of proliferating cells are beneficial [146, 147].

Platinum-based chemotherapy is another example of chemotherapeutic drug targeting mitosis by inhibiting DNA replication. Most notably the drug cisplatin revolutionized the treatment of testicular cancer, which turned a 90% 1 year mortality rate pre-cisplatin discovery into roughly 95% 10 year survival [148]. On other malignancies, the effects have been somewhat limited, and many studies show that cancer resistance towards the drug occurs rapidly. There are also severe side effects due to the toxicity induced by platinum-based chemotherapy [149-151]. Other widely used chemotherapy drugs include anti-microtubule agents and topoisomerase inhibitors which both also inhibit mitotic activity.
A shared trait among many of the old widely used chemotherapeutic drugs is therefore their non-specific nature, as cell division, while being a hallmark of cancer, is by no means specific for malignant cells. Most notably healthy cells that require ample amounts of cell division include cells of the gastrointestinal tract and hair follicular cells. It is therefore no surprise that some of the most common side effects caused by these chemotherapeutic agents are diarrhea, constipation, vomiting, mucositis and hair loss [151-153].

The introduction of radiotherapy was enabled after the discovery of X-rays by Röntgen in 1895 and has been used as therapy against various cancers since then [154]. However, even with advances for over a hundred years, the limitations of radiotherapy remain largely the same. Similar to surgical resection, radiotherapy is unable to distinguish between healthy tissue and malignant tumours and relies on other methods such as imaging or staining techniques to determine treatment location. This means radiotherapy is often considered a supplementary treatment option such as reducing tumour size or burden before treatment with another more specific method, or as primary treatment with curative intention in non-metastatic cancers located where surgery is infeasible. It is also considered a staple of palliative management of late stage cancers. As radiotherapy inflicts considerable tissue damage to the treatment location, it is considered of limited use for curative intentions in metastatic cancer [155, 156].

1.3.4 Prostate cancer

The second most common cancer occurring in men and also the fifth leading cause of death worldwide is prostate cancer with 1,276,106 new cases and 358,989 deaths in 2018 based on Global Cancer Observatory estimates [157]. While many low grade prostate cancers only require active surveillance, a subset of these patients develop metastatic prostate cancer which require intervention [158]. One of the main reasonings for why active surveillance is the modus operandi for prostate cancer around the world is due to the majority of cases being low grade that have limited benefit from treatment, the high incidence of the disease and side effects of treatment potentially being more detrimental than the progression of disease itself [159]. As prostate cancer is often discovered in elderly patients where surgical resection and extensive chemotherapy have considerable risk of complications, this factor is also taken into account when deciding treatment options [160].

For the patients who do develop metastatic prostate cancer, treatment options are often considered mainly life-prolonging rather than curative, and the progression of disease varies considerably. One of the first investigations done specific for prostate cancer is

determining its androgen dependency. Many metastatic prostate cancer cells express androgen receptors which they rely upon for growth signals upon binding. Therefore, androgen deprivation hormonal therapy remains one of the first choice treatment options for metastatic prostate cancer [161, 162]. However, not all metastatic prostate cancers are androgen dependent, and usually even with androgen deprivation therapy, metastatic prostate cancer usually develop resistance over time. This is then usually referred to as castrate-resistant disease. Treatment of castrate-resistant disease usually involves anti-mitotic chemotherapy agents together with radiotherapy for palliative care [163, 164]. Due to the resistance developing nature of metastatic prostate cancer and the mainly life prolonging treatment options today, there is an urgent need for development of curative approaches for this patient group.

1.4 Immunotherapy

As we understand more of the hallmarks of cancer, it is evident that the traditional methods of surgical resection, mitosis inhibiting chemotherapy and radiotherapy have limited effect or severe side effects in treatment of metastatic cancer. While one approach to solve this problem is by simply improving early detection and increase chance of treating cancer in early TNM stages, the feasibility of this approach has its own limitations by sensitivity and specificity of diagnostic methods and the vast amount of different unspecific clinical manifestations [165-167]. As pre-metastatic detection for all cancer is currently unfeasible, better treatment options for metastatic cancer is still required.

An ideal treatment for cancer would involve specific targeting of malignant cells with limited or no effect on healthy cells. As discussed previously the immune system has plenty of evidence both clinically and experimentally that it has the capability of mounting an immune response against cancers, and the methods in which cancer avoids detection by the immune system were also highlighted. Therefore, treatment options aimed at activating the immune system against metastatic cancer while suppressing the immune evasive capabilities of tumours is a promising aspect still under exploration [168, 169].

One of the earliest documented attempts of inducing an immune response against cancer was conducted by Coley in 1891 but utilizing a bacterial vaccine consisting of heat killed bacteria in the treatment of inoperable sarcomas and reported a seemingly beneficial effect on survival for these patients [170]. While the concept of immune surveillance was proposed by Ehrlich in 1909, it took a long time before immunotherapy as a therapy for w-hen immunosurveillance fails re-emerged as a prominent topic in the treatment of cancer. A large contributing factor for this was due to the introduction of radiotherapy and the discovery of tumoricidal effects of chemical weapons used during world war I and II, notably mustard gas and its derivatives, which pioneered the development of the first chemotherapeutic drugs in use and became the “hot topic” for the latter half of the 20th century [171, 172]. During this period, onco-immunologists largely focused on stimulating and enhancing an immune response towards cancer with limited success. The first clinical trial with cytokine treatment utilizing IL-2 started in 1983 and ended with no increased immune reaction against cancer and only side effects in the patients involved [173].

1.4.1 Dendritic cell immunotherapy

The concept of using DC and their capability of inducing an adaptive immune response was pioneered by none other than one of the discoverers of these cells Ralph Steinman [174]. During his final years he was diagnosed with metastatic pancreatic adenocarcinoma in and decided to use himself as a case study for the potential of dendritic cell-based immunotherapy. Convincing his colleagues and collaborators, he was treated with RNA/peptide-loaded autologous dendritic cells in combination with chemotherapy, CTLA-4 checkpoint inhibitor and GVAX (GM-CSF-Gene-transduced allogeneic-irradiated pancreatic cancer cells). Sadly, he died just 3 days before posthumously being awarded the Nobel prize in medicine in 2011, but by then he had survived for four and a half years with a diagnosis that on average kills within a year and only about 1% 5 year survival rate [51, 175].

The potential of dendritic cells have shown great promise in animal models where dendritic cell based immunotherapies have shown capability of inducing tumour

specific CTLs and subsequent remission [176-178]. Dendritic cell based immunotherapy has also shown increased efficiency at eradicating tumours in mouse models when combined with checkpoint inhibitors [179].

Clinical trials involving dendritic cell-based immunotherapies on humans, however, have shown limited efficacy. While there are some phase II trials showing improvement in outcome in metastatic melanoma, phase III trials have largely been inconclusive or ineffective in their outcome [180-182]. What all the clinical trials on DC based vaccines show, however, is that adverse events are limited, and the safety profile is high [183]. Furthermore, many of the trials have clear individual outliers with long term survival, suggesting that while statistically non-significant, it is likely that certain subgroups of patients could benefit greatly from DC immunotherapy. This phenomenon echoes the initial trial results of checkpoint-inhibitors, where overall increase in survival was modest, but certain individuals in the treatment group had long lasting progression free survival [184, 185]. DC based immunotherapy is also marred with lack of standardization between groups. Many trials vary in duration of vaccination, DC concentration of vaccination, location of administration and many other variables. This makes it difficult to compare the results of many of the clinical trials [186]. Other factors that might contribute to the disappointing results of moDC vaccine trials could be how response rate is defined. As many of the trials use statistical methodology from chemotherapy trials where patients often see results within weeks, the delayed but long lasting effect of immunotherapy that might take months and several sessions to work might be overlooked in those clinical trials and therefore new methods are in development to accurately measure the effect of delayed response rate [187].

One of the main hurdles for utilizing DCs for therapy, is their accessibility. Shortly after the discovery of DCs 1973 and shown how potent they were at inducing an adaptive immune response [188], there were attempts of isolating DCs for the purpose of immunotherapy. One of the first feasible methods of getting adequate numbers of DCs in vitro was generating them from their hematopoietic progenitors identified by CD34+ [189]. As those were collected from the bone marrow, feasibility of this

method was limited. It was not until 1994 that a method of generating DCs from monocytes was discovered by stimulating them with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) to generate monocyte-derived DC (moDCs)[190]. This enabled generation of ample amounts of DCs from peripheral blood as around 10% of circulating PBMC comprise of monocytes. While there are some people who argue about the difference between conventional DC and moDC, there are studies that show moDC have comparable or even superior immunostimulatory effect [191].

While moDCs have been shown to be able to trigger an immune response and have shown to be quite potent in vitro, as most clinical trials with moDC shows, there is room for improvement. As moDCs generated from monocytes by IL-4 and GM-CSF are immature, they only have a weak immunostimulatory effect on T-cells, they are not optimal for the purpose of immunotherapy [192]. Upon maturation, however, their immunostimulatory capabilities increase significantly, but what kind of immune response observed depends highly on the 3 signals conveyed to naïve T-cells [51].

Mirroring the function of DCs in vivo, the context of how an antigen is taken up dictates much of the function of moDCs. This is affected by many factors, but some of the most important factors are the cytokines or stimulants used to mature moDC and the duration of stimuli [193-195]. Other factors such as what type of container used, and isolation method used for the moDC also affect their functionality [196]. The lack of standardization between laboratories working on moDC makes it difficult to compare many of the different findings and subsequently hard to determine if the low success rate of DC immunotherapy in clinical trials are due to patient conditions that makes treatment unfeasible, or if the DCs used were inadequate for their purpose. As we understand more about the maturation process of moDCs and the challenges of cancer however, it is evident that some of the more commonly used maturation processes are inadequate for immunotherapy. The common consensus nowadays is that CD8+ TILs are highly important as it is one of the biggest predictive factors for better prognosis, therefore moDC capable of inducing a robust CD8+ response are desirable [197]. This means high expression of MHC class II, co-stimulatory

molecules and a cytokine profile that skews naïve T-cells towards development of tumour specific CTLs where among others, IL-12p70 is quite important [198].

One of the most popular maturation cocktails used was published in 1997 by Jonuleit et al and utilized a combination of IL-1β, IL-6, TNF-α and PGE_2 (Jonuleit cocktail) and as the gold standard of the time, has been uses in many clinical trials [199]. As clinical trials continued to disappoint, however, people started investigating if there are problems with the moDCs generated. One of the major problems with the Jonuleit cocktail stimulated moDC was the low amount of IL-12p70 secreted which is vital for inducing a CD8+ response [200]. It later became apparent that PGE_2 plays a paradoxical role when it comes to moDC maturation, as it provides a key signal to upregulate CCR7 in moDCs which triggers migration towards lymph nodes, but at the same time also downregulates their immunostimulatory abilities [201, 202]. There is also ample amount of evidence suggesting PGE_2 to be an immunosuppressive cytokine in cancer as DAMP signal [203, 204]. Despite the limitations of PGE_2, expression of CCR7 is absolutely crucial for dendritic cells in order to migrate to lymph nodes as several studies show that DC lacking expression of CCR7 fail migrate [205, 206].

The limitations of the Jonuleit cocktail spurred the development of several other alternatives, such as interferon-based, TLR agonist based and polyinosinic:polycytidylic acid (Poly I:C) based ones that are currently being tested both in vitro and in clinical trials [200, 207, 208].

Apart from to the generation method of the moDC, changing site of injection, increasing dosage or frequency of moDC vaccine, and/or addition of adjuvant therapy like IL-2 injection or chemotherapeutic drugs were investigated in hope of triggering an effective immune response against cancer [209-211].

As limited success has been observed in DC based immunotherapy, postulations started regarding whether it was possible to skip the antigen presentation factor all together and instead directly alter T-cells to recognize known onco-antigens. With better techniques in gene transduction with viral vectors and more recently with the rising prominence of genome editing through clustered regulatory interspaced short palindromic repeat associated protein 9 (CRISP/CAS9), direct customization of TCR

has risen in prominence [212, 213]. One such method is the development of chimeric antigen receptor T-cells (CAR-T) which bypasses many of the tolerogenic challenges faced by normal T-cells by artificially inducing expression of TCR with an intracellular domain that leads to direct activation [214]. The nature of custom designing TCR to recognize cancer relies upon that the cancer has a known onco-antigen, which is the reason why CAR-T was initially only approved for B-cell cancers as they often have specific markers such as CD19, CD20 and CD22 [215-217]. However, as often observed with cancer in many clinical trials, long lasting effect has been limited as selection pressure eventually leads to antigen escape by promoting malignant cells not expressing the targeted antigen [218].

1.4.2 The rise of checkpoint inhibitors

It was not until 2009 after the pioneering works of Allison and Honjo on their research on immunological checkpoint molecules that a paradigm shift occurred in the immunotherapeutic approach to cancer. People started to realize that attempting to force an immune reaction without considering the inhibitory factors had limited chance of success [219-221].

Several clinical trials investigating the effects of CTLA-4 and PD-1 inhibitors in treatment of metastatic melanoma were conducted in the early 2000s and the results had immediate impact upon the field of oncology. Compared to conventional chemotherapy, the long year survival rate of grade 4 metastatic melanoma patients improved between 10-20% to over 40% after treatment with various checkpoint inhibitors. A striking feature among the surviving patients is the reduced occurrence of relapse due to development of drug resistance that plagues traditional treatment options for this patient group and while traditional treatment often had severe side effects drastically reducing quality of life, checkpoint inhibitors often have milder side effects in comparison [221-224].

While melanoma was the first success story of check-point inhibitor treatment, clinical trials involving the use of checkpoint inhibitors in other cancers quickly followed with varying results. Shortly afterwards, promising results were observed in non-small

celled lung cancer and renal cancer where checkpoint inhibitors led to significant improvement in survival [225-227].

Since their rise to prominence, there are currently over a thousand clinical trials investigating the effect of checkpoint inhibitors on various metastatic cancers where most of them are combination therapies of various checkpoint inhibitors in combination with each other, or with targeted therapies, chemotherapies or radiotherapies [228, 229]. However, a clear trend is that monotherapy with checkpoint inhibitors does not yield the best survival rates, and that prognostic markers for which patients benefit from checkpoint inhibitor therapies are severely lacking [230]. While there are markers such as PD-L1 expression on tumour that in theory should be predictive for PD-1 and PD-L1 inhibition therapy, the predictive value is still low indicating that just inhibition of checkpoint molecules are insufficient in activating an immune response against cancer in many patients [231].

A clear trend can be observed when it comes to what types of cancer checkpoint inhibitors are effective in. Tumour mutational burden (TMB) observed in tumours is clearly associated with beneficial effect of checkpoint inhibitor therapy [232-234]. It is likely due to increased chance for the immune system to detect tumour-specific antigens which have higher prevalence in cancers with high TMB as shown in figure 9.

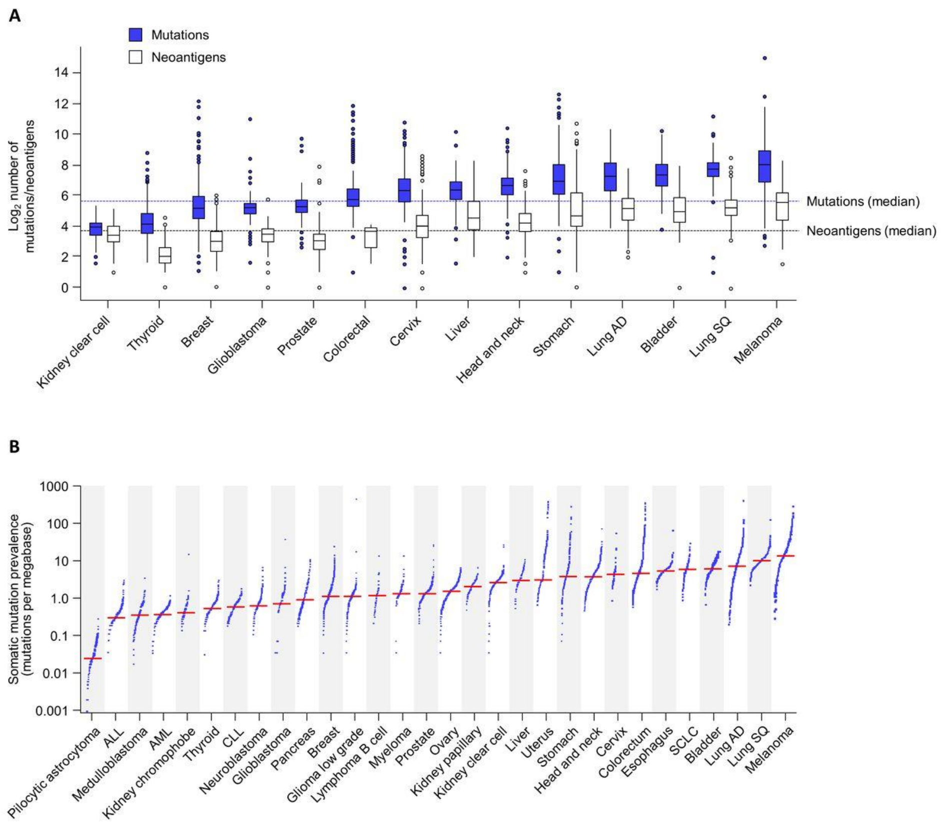

Figure 9. *Tumor mutational burden across several types of cancer*
The figure shows number of mutations compared to amount of neoantigens detected across several types of cancers (A) and the prevalence of somatic mutations across several types of cancer (B). The study by Büttner et al shows that high TMB highly correlates with the two cancers responding most to checkpoint inhibitors being NSCLC and melanoma. Figure from Büttner et al, 2019 [233]. Reprinted with permission from BMJ Publishing Group Ltd.

1.4.3 OK432 in immunotherapy

The concept of using pathogenic components to stimulate the immune system is still widely researched today, and strikingly similar to Coley's work in 1891, an immunostimulatory drug comprising of freeze-dried Streptococcus pyogenes named OK432 was approved in 1975 in Japan after earlier studies conducted by Okamoto et al found anti-cancer activity in haemolytic streptococci [235]. OK432 is currently one of the primary drugs of choice for unresectable lymphatic haemangioma after Ogita et al reported remarkable shrinkage of tumour in over 90% of paediatric patients [236]. As the only side effects appears to be slight fever in a minority of the patients, the drug quickly became a common adjuvant for various ailments in Japan. With the emergence of moDC for immunotherapy, Japanese researchers were quick to postulate the potential of OK432 in stimulating these cells and early research indicated that OK432 matured moDC could induce tumor specific CTLs [237]. Further research a decade later by Hovden et al investigated the mechanisms of how OK432 stimulated moDC and found out it partially acted as a TLR3 agonist and managed to stimulate moDCs into secreting IL-12p70. The main issue with OK432 matured moDCs though was the lack of chemotaxis as they had negligible expression of CCR7 [238, 239].

1.4.4 Challenges of immunotherapy

As discussed previously, while there is potential for DC based immunotherapy and both in vitro and animal studies have shown great promise, it is evident by the clinical trials there are many challenges facing not just DC based immunotherapy, but immunotherapy in general.

As demonstrated clearly by clinical trials involving immunotherapy of various forms, there is a high degree of individual variation on what patients benefit from therapy. A flaw of the common nomenclature is that cancer is an umbrella term for ailments with similar clinical features, but it does not necessarily translate well into immunology as cancer subtypes and heterogeneity differ considerably when it comes to tumour specific antigen expression, tumour microenvironment and direct effect on the immune system [240, 241].

By investigating the effect of various cancers on the immune system, a more precise decision can be made on determining what cancers immunotherapy have higher likelihood to work on. As covered earlier, mutational burden of the cancer type correlates with the outcome of immunotherapy, but for clinical trials the average age of included individuals likely plays a role. It is a well-known fact that immune impairment is more prevalent with age and as most cancers are heavily associated with age, many individuals might have limited benefit from immunotherapy [242]. It has also been shown that for many cancers, moDC generated from patients have impaired functions [243, 244]. Shinde et al reported several distinct properties regarding moDC generated from multiple myeloma patients when compared to healthy controls. While antigen-uptake ability and T cell stimulatory ability was similar to that of healthy controls, moDC from multiple myeloma patients had lower viability, impaired CCR7 expression and altered cytokine profile [243].

Another challenge for immunotherapy is the ethical decisions surrounding inclusion criteria for clinical trials. In theory, DC based immunotherapy has more benefit the earlier it is introduced as to promote the elimination phase of the three Es and minimize the risk of prolonged Equilibrium state and subsequent Escape despite treatment. However, as experimental treatments in general only get approved for patients where all other options have been exhausted, many patients included have had anti-mitotic chemotherapy which impairs the immune system and hampers the effect of immunotherapy long after treatment end [245, 246].

Other precautions are that of side effects. While as mentioned more or less all the clinical trials investigating moDC therapies have been shown to have minimal side effects, as shown by trials combining CTLA-4 inhibitor and PD-1 inhibitor, therapies that are well tolerated by monotherapies can quickly become detrimental once combined without adequately assessing therapeutic windows [247, 248].While animal models have been useful as safety screening for many different drugs, their use for evaluating immunotherapeutic methods needs to be carefully evaluated.

1.4.5 Challenges of immunotherapy in prostate cancer

While immunotherapy in the form of checkpoint inhibitors has become a huge success in cancers like melanoma and non-small celled lung cancer, checkpoint inhibitors have had limited success in prostate cancer trials [249]. There are many factors that make prostate cancer challenging for the immune system. Compared to many other cancers, prostate cancer has relatively low mutagenic burden and limited expression of cancer specific antigens. In addition, metastatic prostate cancer appears to have strong capability of transforming local fibroblasts into CAFs and skew local macrophages into M2 phenotype [250, 251]. While immunotherapy might appear bleak for such conditions, there have been reported case studies where immunotherapy appears to work [252]. There are also clinical trials investigating DC-based immunotherapy efficacy in metastatic prostate cancer where integrity of the immune system is a prognostic factor and trials where similarly to initial results using checkpoint inhibitors in other cancers, see a subset of patients that appear to have long term benefits after immunotherapy [253, 254]. These findings do suggest that while difficult, immunotherapy might be viable for a specific subset of patients.

2. Aims

The overall aim of the project was to increase our understanding of how different culture conditions affect moDC and the potential of OK432 as a maturation stimulus.

The specific aims were:

1: To investigate how OK432 in combination with other compounds affects maturation and T cell stimulatory capacity of moDCs compared to the gold standard at that time, the Jonuleit cocktail

2: To investigate how culture dish surfaces with different adherence properties affect moDC properties regarding phenotype and functionality

3: To investigate if the OK432 based stimulation cocktail is applicable on cells from metastatic prostate cancer patients .

3. Material and methods

3.1 Cohort information

The project was conducted according to the Declaration of Helsinki. All anonymised biological material in the form of buffy coats used for paper I was collected at the Blood Bank of the Haukeland University Hospital after written informed consent was collected. As the study was a technical and methodological development work that used anonymised biological material, no approval from the regional ethical committee was required.

For paper II, freshly drawn peripheral blood was collected from 19 healthy volunteers into BD Vacutainer ACD-A 10 ml citrate tubes (BD, Franklin Lakes, USA). Informed consent was obtained from all donors. Informed consent was gathered from all donors and approved by the regional ethical committee Western Norway (REK Vest; #2009/686).

In paper III, monocytes were isolated by elutriation from leukapheresis preparations of 6 prostate cancer patients included in the clinical trial (Clinical.trials.gov. ACT2001 - NCT02423928; EudraCT Number: 2014-001898-14) in a certified Good Manufacturing practices (GMP) grade lab at The Section of Cell Therapy (Oslo University Hospital, Oslo; Norway). The study was approved by the Norwegian Regional Ethical Committee (REK #64205; REK#2014/1052).

3.2 Blood collection, PBMC and monocyte isolation and cryopreservation

PBMCs were separated by density gradient centrifugation (Lymphoprep™, Axis-Shield, Norway). Monocytes were then isolated using plastic adherence, which utilizes monocytes' tendency to stick to plastic surfaces to separate them from non-adherent cells (NACs) (Paper I & III). For certain experiments fresh blood collected from healthy volunteers were collected in BD Vacutainer ACD-A 10 ml citrate tubes (BD, Franklin Lakes, USA) and PBMCs were separated by density gradient centrifugation.

Monocytes were then separated by negative isolation with Monocyte Isolation Kit II (Miltenyi Biotec Norden AB, Lund, Sweden) which utilizes antibody coated magnetic beads binding to non-monocyte PBMCs running through a magnetic funnel. In 4 of the experiments, anti-CD-61 microbeads (Miltenyi Biotec Norden AB, Lund, Sweden) were also added to reduce platelets (Paper II). DCs were frozen in some of the experiments in X-vivo 20 medium (Lonza) with 10% DMSO in Mr. Frosty freezing containers (both Sigma-Aldrich) at -80 ^{oC} (Paper I & III).

3.3 Cell culture, DC generation and maturation

In paper I, moDCs were generated by culturing isolated monocytes in RPMI 1640 (Lonza) with 10% FCS, 100 units/ml penicillin, 100µg/ml streptomycin, IL-4 (20 ng/ml) and GM-CSF (100 ng/ml) (both ImmunoTools, Friesoythe, Germany) at 37 ^{oC} and 5% CO_2 humidified atmosphere in adherent cell culture plates (Nunclon Δ, Thermo Fischer Scientific, USA; Paper I).
In paper II, moDCs were generated by culturing isolated monocytes in CellGro DC medium (CellGenix) in both adherent and non-adherent culture plates (Nunclon Hydrocell & Nuncleon Sphera, Thermo Fischer Scientific, USA). In paper I and paper II, the cells were cultured for 3 days with replenishment of IL-4 and GM-CSF on day 2, while in paper III, thawed moDCs from prostate cancer patients were cultured in Cellgro DC medium in same concentration of IL-4 and GM-CSF for two days. One hour prior to maturation stimuli, 1 µg/ml tuberculin-purified protein derivate (PPD, Statens Serum Institut, Denmark) was used as recall antigen (Paper I & II & III) before stimulation with various stimulation cocktails as shown in table 1. As all blood was collected from residents in Norway who had mandatory Bacillus Calmette-Guèrin Vaccine (BCG) vaccination from 1948-1994 and all donors/patients were in that age group, PPD was deemed fit as recall antigen [255]. After maturation, moDCs were used for co-culture or for analysis on an LSRFortessa (BD Biosciences) flow cytometer.

Table 1. Monocyte-derived dendritic cell stimulation cocktails used in the different experiments

Cocktail name	Content	Paper
Jonuleit cocktail	IL-1β (10 ng/ml, Immunotools) IL-6 (1000 U/ml, Immunotools) TNF-α (10 ng/ml, Immunotools) PGE_2 (1 µg/ml, Sigma-Aldrich)	I & II
LPS	LPS (100 ng/mg, Sigma-Aldrich)	II & III
OK432	OK432 (0.1 KE/ml, Chugai Pharmaceutical Co. Ltd)	I
OK432 + CL097	OK432 (0.1 KE/ml) CL097 (1 µg/ml, Invivogen)	I
OK432 cocktail	OK432 (0.1KE/ml) CL097 (1 µg/ml) PGE_2 (0.5 µg/ml)	I & III
Lövgren cocktail	TNF-α (10 ng/ml) IFN-γ (1000 U/ml, Sigma Aldrich) R848 (2.5 µg/ml, Invivogen) PolyI:C (20µg/ml, Sigma Aldrich)	I & III
Kalinski cocktail	TNF-α (50 ng/ml) IL-1β (25 ng/ml) IFN-α (3000 U/ml, Immunotools) IFN-γ (1000 U/ml) PolyI:C (20µg/ml)	I
Zobywalski cocktail	TNF-α (10 ng/ml) IL-1β (10 ng/ml) IFN-γ (5000 U/ml) PGE_2 (0.25 µg/ml) R848 (1 µg/ml) PolyI:C (20 ng/ml)	I
IL-10	IL-10 (10 ng/ml; Immunotools)	II
DexVD3	Dexamethasone (1 µM; Sigma Aldrich) 1α, 25-Dihydroxyvitamin D3 (VD3) (1 nM; Enzo Life sciences)	II

3.4 Blocking antibodies

In paper II, blocking antibodies against CD11a, CD11b, CD11c, CD18 or E-cadherin (all 10µg/ml; Invitrongen/Thermo Fisher, USA) were added to moDC culture during generation to investigate the effect of those surface molecules on cell to cell and cell to culture dish adhesion. Morphology was analysed by light microscopy using a Cytation 5 Cell imaging-reader (BioTek instruments).

3.5 moDC – NACs co-culture

In paper I and II, mixed leukocyte reaction (MLR) co-culture was performed. After generation of moDC, co-cultures with either autologous or allogeneic NACs were setup in X-vivo 20 medium with addition of IL-2 (50 U/ml) and IL-7 (10 ng/ml; both Immunotools) in Nunclon Δ well plates and incubated at 37 °C and 5% CO_2 humidified atmosphere for 5-7 days. Some of the NACs were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen) according to manufacturer's instructions (papers I & II). After culturing, the NACs were used for IFNγ secretion assay and/or analysis on an LSR Fortessa or Accuri C6 (BD Biosciences) flow cytometer.

MLR is a method to measure T-cell proliferation capacity of moDC through co-culture. In an allogeneic co-culture, the MHC molecule will be recognized as foreign and trigger clonal expansion of recipient T-cells, but in autologous co-culture, clonal expansion is not triggered without a stimulatory antigen presented by the APCs (Figure 10)

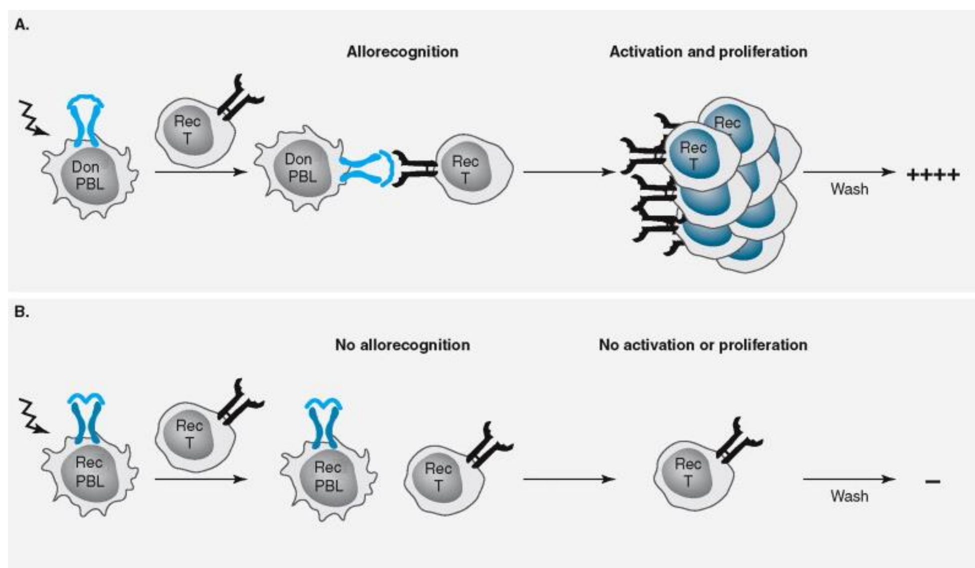

Figure 10. *Allogeneic mixed leukocyte reaction*
Antigen presenting cells of donor allogeneic peripheral blood lymphocytes (PBL) have their MHC molecules (blue) recognized by recipient T-cells triggering activation and proliferation (A), but autologous MHC does not get recognized by recipient T-cells and no activation or proliferation occurs (B). Figure from The Immune response: basic and clinical principles, T. Mak and M. Saunders, [256], modified with permission from Wolters Kluwer Health, Inc.

3.6 Flow cytometry

To analyse the phenotype of the generated moDCs and induced T-cells, flow cytometry was utilized. Flow cytometry is a commonly used tool both in research and clinic to measure and analyse large amounts of cells and other particles in a suspension an accurate manner. The strength of the method lies in its ability to measure multiple features on an individual particle in a heterogenous mixture. Flow cytometry utilizes hydrodynamic focusing to force particles to pass in a single file through the laser beam. Particles are generally labelled with fluorochrome-attached antibodies or fluorescent dyes specific for a target of interest, for example a cell surface receptor.

The light scatter from the laser beam hitting the particle or fluorescence emitted by excited fluorochromes is converted into electronic signals by photomultiplier tubes (PMTs). These electronic signals from each particle can then be analysed. The use of multiple lasers and optical filters allows excitation and detection of emission at different wavelengths for the identification of different fluorochromes and hence targets (cell receptors etc.) by their unique excitation and emission spectra simultaneously. Expression levels of targets can then be quantified, or alternatively the combination of identified features can be used to identify cell subsets. A typical flow cytometry setup for moDC is shown in figure 11. Fluorochrome panel design and protocols were determined by study purpose and antibodies were titrated to minimise non-specific staining. While one or two study specific markers were included or excluded, in general the analysed markers comprise of signal 1 marker HLA-DR, signal 2 markers CD80, CD83 and CD40, maturation marker CD83, chemokine receptor CCR7 and checkpoint markers PD-L1 and PD-L2 with some additions depending on the study [238, 257-259]. The whole list of markers used is shown in Table 2 and Table 3 for moDC and T-cells respectively.

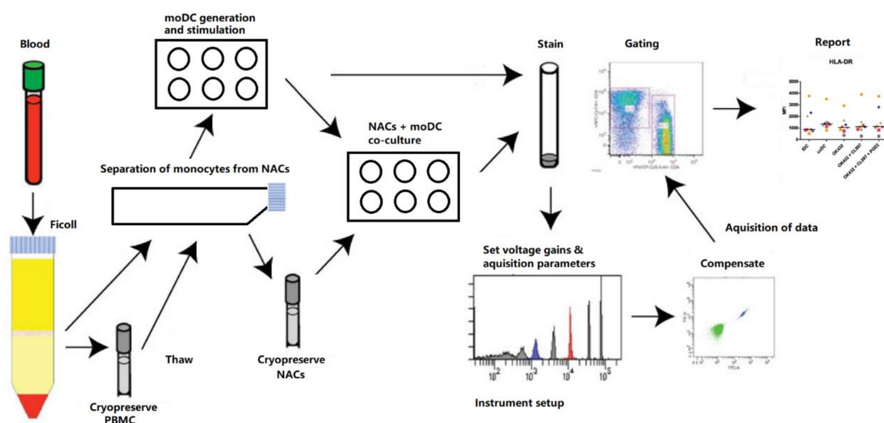

Figure 11. *Typical flow cytometry experiment on moDC isolated by plastic adherence. PBMC can be isolated through density gradient centrifugation with Ficoll and cryopreserved or cultured. On adhesive surface conditions, monocytes will adhere to culture container at 37 °C, 5% CO_2 humidified atmosphere while NACS will remain suspended. The monocytes can then be generated into moDC with or without maturation stimuli. The resulting moDC can then be co-cultured with NACs or stained and analysed. NACs after co-culture with moDC can also be stained and analysed. The flow cytometer is setup to optimize detection of individual fluorochromes and minimize run-to-run variation by adjusting voltages of PMTs. Analysis is subsequently performed by various software available, spectral overlaps of fluorochromes are corrected (compensation) and cell subtypes are identified by their antigen expression indicated by their respective fluorochrome in a process called gating. Figure inspired by Maecker et al [260]. Reprinted and modified with permission from Springer Nature.*

Table 2. Monoclonal antibodies with fluorophores used for moDC panel in the different projects

Fluorochrome	Surface marker	Clone	Used in paper
FITC	CD14	18D11	I + II + III
PE	CD1a	HI149	I + II + III
PE-Cy7	CD40	5C3	I + II + III
Alexa Fluor 647	CD86	IT2.2	I + II + III
Brilliant Violet 421	CCR7	G045H7	I + II + III
PE-CF594	CD83	HB15e	I + II + III
Horizon V500	HLA-DR	G46-6	I + II + III
Brilliant Violet 605	CD80	2D10	I + II
PerCP-Cy5.5	CD38	HIT2	I + II
FITC	CD18	TS1/18	II
PerCP-Cy5.5	CD11c	3.9	II
APC-Cy7	HLA-A,B,C	W6/32	II
PE-Cy7	PD-L1	MIH1	II
Alexa Fluor 647	PD-L2	MIH14	II
Brilliant Violet 421	CD11b	ICRF44	II
Brillivant Violet 785	CD80	2D10	III
Brilliant Violet 711	PD-L1	29E,2A3	III
APC-Cy7	PD-L2	MIH18	III
PerCP Cy5.5	4-1 BBL	5F4	III

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Table 3. Monoclonal antibodies with fluorophores used for T-cell panel in paper III

Fluorochrome	Antibody	clone
AF488	CD20	2H7
AF488	CD56	NCAM1
PerCP-Cy5.5	CD8	RPA-T8
PE	CD28	CD28.2
PE-Cy7	CD127	A019D5
PE/Dazzle594	CD137	4B4-1
APC-Cy7	CD4	RPA-T4
AF700	CD3	UCHT1
APC	CCR7	G043H7
BV785	CD45RO	UCHL1
BV605	CD45RA	HI100
BV650	CD25	BC96
BV421	CTLA-4	BNI3
BV711	CCR5	3A9
BV510	PD1	EH12.2H7

3.7 Chemotaxis assay

In paper I, moDCs were added to the upper chamber of an 8µm transwell membrane 96-well plate (Corning Lifesciences) and left to migrate towards CCL19 (100 ng/ml, Immunotools), a ligand of CCR7, in X-vivo 20 medium for 4 h at 37 °C, 5% CO_2 humidified atmosphere before quantification with a CASY cell counter (Roche) which uses a low voltage field across a medium to detect cell size by electric resistance. As membranes act as electric insulators, detection pores in the machine registers the resistance encountered by the electric field to measure the size of cells. As dead cells are often either very small or have broken membrane integrity, their signals are considerably smaller than those of living cells. The method can also be used to differentiate small cells from large cells.

3.8 IFN-γ secretion assay

Analysis of T-cell activity by measuring their IFN- γ secretion was performed in all papers. After 7 days moDCs and NACs co-culture with or without 5 days resting period, some NACs were isolated and cultured with mature autologous moDCs with or without PPD loaded for 16 hours in X-vivo 20 at 37 °C, 5% CO_2. The IFN-γ secretion was then measured using an assay kit (Miltenyi Biotec). The method utilizes conjugated antibodies bound to surface molecules on T-cells with a receptor for IFN- γ which it catches upon secretion by its bound cell. The caught IFN-γ is then bound by a detection antibody with fluorochrome attached and subsequently analysed on a flow cytometer. CD4 and CD8 antibodies were used to distinguish the cells from each other. T-cells stimulated with Staphylococal enterotoxin B from Staphylococcus aureus (SEB), a superantigen, was used as positive control, while co-cultures with moDC without PPD loaded was used as negative control.

3.10 ELISA (Paper I)

Cell culture supernatant was stored at -20 °C. To quantify IL-12p70 and IL-10 secretion into the supernatant, sandwich enzyme-linked immunosorbent assays (ELISA; BioLegend) were utilized according to the manufacturer’s instructions. The samples were run in technical duplicates to reduce variability. The concept uses a capture antibody coated on a well plate and subsequent binding with a biotinylated detection antibody. Horseradish peroxidase (HRP) is then added to form an Avidin-Biotin complex which reacts with 3,3’,5,5’-tetramethylbenzidine (TMB) substrate which in turn results in a measurable colorimetric reaction that can be quantified by a microplate reader. Concentrations of antigen in medium are determined by comparing with a parallel-run standard curve.

3.11 Luminex assay

Cytokine and chemokine concentrations from culture supernatant after moDC maturation were determined using a 25-plex Luminex assay cytokine and chemokine panel (Invitrogen, catalogue number LHC0009) and run on a Luminex 100 System (Luminex Corporation, Austin, TX) according to the manufacturer's instructions (Paper I & II).

The method utilizes polystyrene beads with unique tagged fluorochromes, biotinylated antibodies and Steptavidin/Phyocerythrin to detect cytokines or chemokines. Similar to ELISA, the concentrations are then calculated from a standard curve.

3.12 Data analysis

Visualization and analysis of flow cytometry data was done using Flowjo (Tree Star). Identification of cell subsets were done using light scatter properties in combination with expression levels of different receptors, and various controls including unstained cells and Fluorescence Minus One (FMO). Statistical analysis of measurements was performed in Prism 5 (Graphpad). Significance between samples were tested using Kruskal-Wallis one-way analysis of variance (ANOVA) test (paper I & III) or 2way ANOVA test (paper II) with Dunn's post-test (paper I & II & III) or Bonferroni post-test (paper II). All statistical formulas and post-tests used are for non-parametric analysis due to individual variation of the immune system and the low amount of study subjects. Significance value was set to $P < 0.05$.

4. Summary of the main results

Paper I

Different maturation stimuli comprising of, OK432 alone, OK432 + CL097 and OK432 cocktail were tested on the generation of moDC from healthy donor donors and was compared to the Jonuleit cocktail. Surface molecules HLA-DR, CD83, CD1a, CD80, CD86, C40, CD38, CCR7 and CD14 were measured by flow cytometry to determine phenotype. All cells showed high expression of HLA-DR. All maturation cocktail stimulated cells showed increased maturation marker CD83 compared to unstimulated control, and high expression of co-stimulatory molecules CD80 and CD86. OK432 stimulated cells had low expression of CCR7 but was elevated above expression by Jonuleit stimulated cells with the addition of CL097 and PGE_2. OK432 cocktail was also the most capable of inducing CD80 and CCR7 among the cocktails tested.

Migration assay showed increased migratory capacity by moDCs stimulated with PGE_2, and almost no migratory activity was seen in moDCs matured without PGE_2. Jonuleit matured moDCs showed highest amount of transwell migration while OK432 cocktail matured moDCs had around half as much but significantly higher than controls.

To measure cytokines, Luminex multiplex analysis of 26 different cytokines was performed. All OK432-matured DCs had a significant increase in IL-12p70 secretion compared to Jonuleit cocktail and immature control that was further elevated with the addition of CL097 but decreased by PGE_2. This pattern was also observed with RANTES/CCL5, MIP-1α/CCL3, and MIP-1β /CCL4. IL-12p40 was highly elevated by OK432 cocktail, but not statistically significant compared to the other matured DC populations. IL-10 secretion was detected at low levels. IL-8 secretion was significantly reduced by the addition of OK432. IL-7 was increased in every matured DC population. IL-17 secretion was increased by OK432 alone but decreased with the addition of CL097 or PGE_2. IL-16, IL-1RA, IFN-γ, IFN-α, MCP-1, IL-2R, and IL-13 secretion did not show obvious differences between sample groups. IP-10, IL-2, IL-5,

IL-13, or Eotaxin was secreted at very low levels while IL-4, GM-CSF, IL-1β, IL-6, and TNF were excluded from the analysis as they were used in the culture media.

MLR was performed to investigate T-cell stimulatory capacity. OK432 stimulated moDCs were superior to Jonuleit cocktail stimulated moDCs at inducing T-cell proliferation with OK432 cocktail stimulated moDCs being the most potent. IFN-γ assay was used to determine antigen-specificity of those expanded cells and showed that while OK432 cocktail stimulated moDCs were able to induce antigen-specific T-cells, it was not significantly more than Jonuleit cocktail stimulated moDCs.

To compare OK432 based maturation stimuli with other more recent maturation cocktails, an IL-12p70 ELISA was performed on moDCs stimulated by Jonuleit cocktail, OK432 + CL097, OK432 cocktail, Kalinski cocktail, Lövgren cocktail and Zobywalski cocktail. All cocktails induced higher IL-12p70 secretion compared to Jonuleit cocktail with Lövgren cocktail inducing significantly higher secretion than the rest. OK432 based cocktails induced similar level of IL-12p70 secretion to the other cocktails otherwise.

Paper II

The effect of surface adherence on the generation of moDC was investigated by comparing generation on standard cell culture dishes and non-adherent culture dishes. Two immunogenic conditions comprising of LPS and Jonuleit cocktail stimulated moDC and two tolerogenic conditions comprising of IL-10 and DexVD3 stimulated moDC were tested on the different surfaces.

In all conditions generated on non-adherent surface culture dishes, homotypic clustering could be observed by light microscopy. Several blocking antibodies were tested against known molecules associated with adhesion, CD11a, CD11b, CD11c, CD18 and E-cadherin. Blocking CD18 resulted in reduced homotypic clustering while blocking CD11a or CD11b appeared to promote homotypic clustering.

Phenotype under the four different conditions was investigated by flow cytometry with a panel of 15 different surface molecules. The results showed that all surface molecules were influenced by the culture dish used. Particularly interesting,

checkpoint molecule PD-L1 was decreased on moDCs stimulated with the Jonuleit cocktail in a non-adherent culture dish while PD-L2 was increased. The differences were noted to depend both on culture dish adherence and stimuli.

Cytokine production was investigated using a 25-plex Luminex platform and showed cytokine production varied depending on the treatment and the surface used. In general, LPS-DC had the highest production of most cytokines. Most cytokines were overall secreted at lower levels on non-adherent surface. While the cytokine profile of the DC cultured in tolerogenic conditions as well as control showed limited differences in cytokine profile regardless of culture dish surface, the LPS stimulated DCs had significant profile differences. IL-10 and TNF-α secretion was increased in all LPS stimulated DCs on standard cell culture dish compared to non-adherent surface, while the opposite was true for IL-15 and MIG.

In order to investigate the possibility that isolation impurities might have an impact on the clustering, phenotype, and cytokine production, four of the monocyte isolation procedures were performed using additional anti-CD61 beads to remove platelets. Both the phenotype of the generated DC populations and their produced cytokines did not show any clear differences between the preparations with and without residual platelets

Lastly, the T cell stimulatory capacity of the generated moDC populations was analysed in an allogeneic MLR. No obvious differences were observed between the different surfaces, regardless of the DC population used as stimulator. The proliferation of monocyte-depleted PBMC co-cultured with the tolerogenic moDC populations was less than with immature DC. The immunogenic moDC populations was further analysed in an autologous setting with an IFN-γ assay. Both LPS- and Jonuleit-cocktail stimulated cells were able to induce antigen specific autologous CD4+ and CD8+ T cells, with slightly higher numbers of IFN-γ producing T cells upon using LPS-stimulated DC, independent of the culture dish used.

Paper III

To investigate the potential of OK432 cocktail matured moDC on T-cells from cancer patients, moDCs were collected from patients with metastatic prostate cancer and stimulated with either OK432 cocktail, Lövgren cocktail or left untreated as negative control. Phenotype of moDC was investigated using a panel of 11 different surface markers. All analysed moDC populations yielded phenotypically mature DC with high expression of MHC class II, co-stimulatory molecules CD40, CD80 and CD86, and moderate expression of maturation marker CD83 including the negative control. Checkpoint molecules PD-L1 and PD-L2 were also highly expressed. In contrast to paper I, using moDC from healthy donors, the addition of PGE_2 in the cytokine cocktail did not positively affect CCR7 expression.

Phenotypic analysis with a 15-marker panel showed that T-cells co-cultured with the OK432 cocktail stimulated moDC for 7 days significantly increased the proportion of CD25+/CD45RO+ cells among both CD4+ and CD8+ cells. This population was also relatively low in CD127 expression. No significant difference was observed in checkpoint molecules CTLA-1 or PD-1.

IFN-γ secretion assay was used to investigate antigen-specific T-cell activity, but large interindividual variations and low number of samples make significance hard to interpret. However, IFN-γ secretion by T-cells was increased after stimulus by PPD loaded moDC compared to stimulus with moDC without PPD loaded in all samples suggesting that it is possible to induce antigen-specific reaction in T-cells from metastatic prostate patients.

5. Discussion

5.1 Methodological considerations

5.1.1 Culture, treatment and storage condition of cells

Many laboratory and logistical methods can influence the phenotype of PBMCs and their behaviour. This has been shown in several studies where investigators found out that PBMC varied considerably depending on factors such as storage time, storage concentration, whether it is whole blood or buffy coats, temperature, workflow and cryopreservation [261].

In particular, yield after cryopreservation differs considerably between methods, but just higher viability does not necessarily mean best preservation method as there are studies showing that freezing medium can significantly alter the cell functions [262]. Generated moDCs are no exception to this as shown in an earlier study [263].

Medium used for cell culture in vitro is considerably different than in vivo. Conditions such as natural physiological changes, cell to cell communication, physical and chemical conditions are all altered. It is known that too high or too low concentration of cell culture can affect cell functions [264].

Culture duration was also a consideration during our study design. Different groups often use different protocols on how long monocytes should be cultured with IL-4 and GM-CSF and how often the cytokines should be replenished [200, 265, 266]. In our group we determined on preliminary results that after 3 days, the cells had drastically reduced CD14 expression, a monocyte marker, and highly elevated HLA-DR and CD1a used as markers for moDC differentiation which is in line with a study by Schendel et al [266].

While working with cells, mechanical stress from centrifugation, pipetting and temperature differences can lead to cell apoptosis or necrosis and yields in the various experiments varied considerably. Many immune-cells are naturally sensitive to such conditions due to having DAMP receptors and can be stimulated to secrete various cytokines as a result [267]. While one strives to keep treatment equal between

samples, practically it is not possible as the samples themselves are not equal in terms of concentration upon sampling, stickiness in the case of monocyte adherence, and other factors. However, if the differences are large enough, conclusions can be drawn even with slight margins of error.

5.1.2 Considerations in moDC stimulation

As one of the main investigative aims of the project, OK432 with its TLR3 stimulatory capability along with other factors was considered a prime candidate for moDC stimulation in cancer immunotherapy. The rationale behind utilizing OK432 over a TLR3 ligand is due to the nature of what OK432 is, which are freeze dried Streptococcus particles, which means it can stimulate immune cells more than just in a TLR3 dependent manner. The hope is that OK432 can stimulate immune cells into interpreting cancer antigens as pathogenic by proxy. Due to the long history of OK432 used clinically in Japan as adjuvant for various cancers, it has shown to be a safe substance to inject into humans, and by extension cells cultured with it.

The addition of CL097, also known as Imidazoquinoline, as an TLR7/8 agonist was to improve the immunogenic stimuli on moDC further. As it has been used in dermatological products since 1997 where the first Imidazoquinoline creams were approved by the FDA and its safety profile is well known as a skin product [268].

5.1.3 Special considerations on MLR

In several of our experiments we used MLR to measure T-cell stimulatory capacity. To investigate the T-cells ability to undergo clonal expansion, PPD was picked as recall antigen. Due to Norway’s vaccination programme, it is to be expected that donors have memory cells specific for PPD, and therefore proliferate upon successful presentation by APC. This gives valuable information about the quality and properties of the APCs but does not represent presentation of cancer neoantigen and ability to react towards that. As knowledge about choice of cancer antigens is limited and patient availability is limited, investigating the APC properties of moDC on a recall antigen was deemed adequate for initial impression.

The nature of MLRs also might affect the analysis as proliferation means conditions are not constant in each population. Notably we had to change medium and replenish cytokines to avoid apoptosis by lack of survival stimuli. While the fewer proliferating samples were treated at the same time as the highly proliferating samples, what effect the temporary reduction in survival cytokines and nutrients had is unknown.

5.1.4 Special considerations on flow cytometry

In flow cytometry, there are some error potentials that are hard to avoid. To keep cells in a single file by hydrodynamic focusing, the speed of acquisition matters considerably. Too high acquisition speed leads to risk of doublets, which means two cells passing the laser at the same time and resulting in data error. While this can be mitigated to some degree with adjusting the speed and good gating, it does lead to some slight error. The staining procedure can be time sensitive as fluorochromes naturally loses fluorescence when exposed to light. This can be mitigated to some extent by keeping the stained samples away from or blocking light, but not always feasible. The choice of fluorescent antibodies and the concentration of the staining procedure also matters greatly. Titration of antibodies and adjustment of voltages prior to analysis are crucial for accurate measurement of data. For paper III this became an issue as patient samples were very limited and varied, therefore titration was done on blood from healthy donors which had some differences. This becomes more complex the more fluorochromes included as there are limits to the span of the electromagnet spectrum and fluorochromes that can fit in a single panel. When two fluorochromes are close to each other on the electromagnetic spectrum, overlap can occur. This is something we encountered with some of our fluorochromes, and some concessions had to be made to reduce spillover. Limiting spillover is a fine balance between adequate separation between positive and negative signals as too high signal usually means more spillover, while too low signal means it becomes difficult to separate positive from negative. Mitigating spillover requires compensation, which is a mathematical method of subtracting overlap signals from the respective channels, but it depends heavily on the quality of positive and negative controls, autofluorescence of cells and correct gating [269]. We used OneComp eBeads (eBioscience) stained with antibodies

for fluorochrome compensation and unstained cells for autofluorescence compensation, but slight differences can still be made in compensation calculation. The choice of staining condition also matters as binding happens faster in higher temperature due to thermodynamic activity. However, higher temperature leads to more internalization of surface receptors in T-cells which can affect the analysis. Therefore, we stained T-cells for 30 minutes on ice while moDCs were stained in room temperature for 10 minutes. As dendritic cells are sufficiently large that they appear as a clear population compared to debris and other cells in forward scatter and side scatter plots in flow cytometry, gating around them is not a difficult task as dying or dead cells are clearly smaller. This is harder to gate around regarding T-cells which are smaller and therefore harder to separate from debris and dead cells. Therefore, we used Pacific Orange as a live-dead stain.

5.1.5 Special considerations on migration assay

For chemotaxis assay, transwell methods, while easy to setup, have limited relevance physiologically as concentration of target chemoattractant differs from physiological response and the static nature of wells hardly represent the vascular or lymphatic system. While it does convey information about cells ability to migrate through a membrane, how reflective it is of behaviour in vivo is highly debatable [270]. The choice of using a CASY cell counter was also due to feasibility, but as the purpose was to show that the cells could migrate and controls were not supposed to migrate at all, the slight variance in counting was deemed of little relevant.

5.1.6 Special considerations on IFNγ assay

Measurement of T-cell using an IFN-γ based assay is a reliable way of detecting the number of activated T-cells. Several studies show that antigen-specific T-cells indeed starts secreting IFN-γ upon activation [271-273]. The ratio between moDCs and T-cells are quite important for this measurement, and while the start ratio is similar for all samples, the end ratio can differ based on individual variations due to apoptosis or proliferation which in turn can affect the results, but this is likely not significant due to the short amount of co-culture time (18h). There are concerns that the antibodies

binding to cells can affect them to some degree, but according to the developers of this method it has no negative impact on the cells [273, 274].

5.1.7 Special considerations on ELISA

For ELISA, the concentration of cytokines frozen from supernatant is influenced by the number of cells in the culture. As individual variation is observed when it comes to viability during the generation of moDC and subsequent yield of them, it is no surprise that total cytokine profile of the culture will be affected. As we are comparing maturation treatment of moDC, there should be variations on how immune cells of individuals react to the different cocktails and subsequently affect the total cytokine profile. We can therefore only report how the treatment acts upon the cells as a group rather than how it affects individual cells. To correct for this, results from both ELISA and Luminex are compared to viability data during cell harvesting. As proteins degrade over time and with increased temperature and ELISA is a lengthy procedure, some loss is to be expected during the processing even if being kept on ice for as long as possible [275]. Many of the same limitations can be applied to Luminex assays as well.

5.1.8 Special considerations on statistical analysis

For statistical analysis, the methods picked are appropriate for the studies. However, due to the low number of included samples it can be difficult to interpret. Outliers can heavily skew the grand mean and can mainly be mitigated by larger sample size which is not always possible due to cost or available study population. While trends can be observed even with small sample sizes, one should be careful to draw conclusions even with statistical significance.

5.2 Implications of research results

As clinical trials using moDC show that results are varied and often negative in immunotherapy, and generation methods of moDC vary considerably between labs, investigating what influences the generation process becomes crucial. While steps towards better reproducibility and standardisation is underway with fully automated cell generation methods such as CliniMACS Prodigy by Miltenyi Biotec, availability

to such equipment is currently limited. While generation of immature moDC is generally agreed upon to rely on the method established by Sallusto and Lanzavecchia in 1994 [190], this is not the case for maturation methods. For a long period of time, the maturation cocktail developed by Jonuleit et al has been used for many clinical trials but was deemed insufficient for the purpose of immunotherapy. As referred to previously, many groups have developed their own cocktails for the maturation process of moDC, and how the blood/PBMC is processed and stored also vary significantly. As there is no standard on how to mature moDC for the purpose of immunotherapy, we set out to investigate potential new cocktails and other factors and compare them to that of other protocols. As CCR7 expression and migration to lymph node are crucial for adaptive immune response, our focus was finding a cocktail that both induced CCR7 expression in moDC as well as immunogenic phenotype. As our studies have shown, moDCs are heavily affected by various factors that might be overlooked normally during generation and therefore explain many of the variable results seen in the clinical trials. The burst of excitement generated by the success of checkpoint inhibitors and how it mainly works in specific cancer types and subset of patients has spurred numerous clinical trials on the basis that the methodology is not at fault, but rather the target population. Many of the clinical trials with moDC has followed the same philosophy with disappointing results. Hopefully our research can contribute to better understanding and possible standardization on moDC generation methodology.

5.2.1 The role of OK432 in moDC maturation and the effects of PGE2

As one of our goals was to determine OK432 matured moDC as candidate for immunotherapy, the properties of the OK432 matured moDC had to be compared to the gold standard at the time and prominent cocktails used by other groups [199-201, 207]. As our results show, the cells have properties that are desired for immunotherapy which are high expression of HLA-DR, costimulatory molecules CD80 and CD86 and secretion pro-inflammatory cytokine IL-12p70.

The main problem for OK432 matured moDC as described previously by Hovden et al [238], was the lack of CCR7 expression which meant that moDC matured only with OK432 would in theory not migrate towards lymph nodes and therefore have limited clinical value. As the Jonuleit cocktail managed to mature moDC with high expression of CCR7 but limited immunogenic properties, several groups started investigating which component in the cocktail contributed to this phenomenon. It was subsequently determined that PGE_2 was crucial for CCR7 expression in moDC but was also immunosuppressive [202, 204]. As CCR7 expression is crucial for DC migration to lymph nodes, we hypothesized that the stimulatory and inhibitory functions of PGE_2 might be concentration dependent and that the Jonuleit cocktail used too much of it. Strikingly in paper I, we observed that migratory capability was almost linearly correlated with the amount of PGE_2 added to the maturation cocktail. The results of our finding support the notion that PGE_2 is a key up-regulator of CCR7 for moDC. Why this apparent paradox role exist requires further research, but it might play a role in local inflammatory homeostasis. As shown by Hangai et al [204], PGE_2 is released by apoptotic cells which in turn inhibits inflammatory conditions. This could mean that PGE_2 in high concentration acts as a DAMP and that DCs might mature with more tolerogenic properties without adequate PAMP stimuli. This is in line with what we know about macrophages who change phenotype towards immunosuppressive and regulatory M2 phenotype in the presence of PGE_2 [276]. A clinical support for this hypothesis is observed in pregnancy, an immunosuppressive state, where serum PGE_2 is elevated [277].

The main notable difference between OK432 matured moDC compared to Jonuleit cocktail matured moDC was in their cytokine profile, which showed that OK432 matured moDC had a much more promising profile. The high secretion of IL12p70 is already a good indicator, but interestingly one of the most obvious differences was observed in IL-8 which was significantly reduced for every sample included. Initially as this was part of the 25 Luminex panel, it was not deemed one of the cytokines of interest, but subsequent investigation into literature showed that elevated IL-8 is associated with reduced clinical benefit from checkpoint inhibitors in cancers [278].

Furthermore IL-8 is a chemokine that mainly attracts neutrophils, which in turn might have a negative effect for the purpose of immunotherapy as neutrophil infiltration in tumours appears to be a negative prognostic marker in contrast to CD8+ TILs [120, 279]. This negative role is supported by observations that recruitment of neutrophils to lymph nodes can have an inhibitory effect on the adaptive immune response in cancers [280]. Therefore, the observed low IL-8 secretion by OK432 moDC appears to be a positive trait for the purpose of immunotherapy. It should also be noted that all OK432 matured moDC had elevated IL-12p70 and IL-12p40 compared to Jonuleit cocktail matured moDC. The addition of CL097 appeared to increase IL-12p70 with OK432, but did not affect secretion of IL-12p40 suggesting CL097 to primarily be an proinflammatory stimuli. The addition of PGE_2 significantly increased secretion of IL-12p40 when added onto OK432 with CL097 but also resulted in less IL-12p70. This suggests that PGE_2 might be partially responsible for inactivating IL-12p70 into its inactive form IL-12p40.

5.2.2 Cell culture surface should not be ignored

Commonly observed in many papers involving cell culture, the flasks and containers used for culture are not stated. It has often been taken for granted that in vitro conditions are reflective on in vitro conditions. The established process of erythrocyte and plasma transfusion and the safety profile of those clinical methods add to the notion that cells do not change significantly during transport or processing. In fact procedures such as freezing plasma for transfusion are considered safe, and for military personnel, frozen erythrocytes are also in for clinical use [281, 282]. This however might be limited by the functions of the components as erythrocytes and platelets have limited role outside of their primary functions. As the immune cells are highly sensitive to their environment and have drastic different functions depending on the context of what they detect, the same assumptions cannot be made. It is known that immune cells have homing and anchoring capabilities such as macrophages to specific tissues, and that they have stretch sensitive cellular components that alter their functions [283-285]. It is not hard to imagine that the adherence of cell surfaces can impact the functions of immune cells that are known to adhere to tissue.

In paper II, we showed that adherence plays a significant role in how moDC mature under different stimuli. Interestingly homotypic clusters could be observed in all conditions without adhesion to plate surfaces and this persisted for days, suggesting that monocytes and moDC actively seek adherence to other cells or surfaces. We also showed that blocking CD18 drastically reduced this phenomenon while blocking CD1a and CD1b increased the homotypic clustering which resulted in the hypothesis that CD1a and CD1b might act as a competitive inhibitor to the adhesive functions of CD18 which is in line with a study by Sàndor et al [286].

In addition, we showed that cell phenotype of generated moDC is also affected by culture surface conditions especially in inflammatory conditions. Possible explanations to this might be due to the contextual nature of immune cells in general, as cells often use adhesion signals to contextualize their location and surroundings. Monocytes do not normally reside in tissue, but are instead circulating in the bloodstream or in the spleen and differentiate into other cells once migrated into other tissues [287]. Therefore, it is sensible that their differentiation status differs whether they are attached to a surface or not. How cell to cell adhesion affects their differentiation function, however, remains to be seen as we have yet to investigate the effects of CD18 blockage on moDC generation. There is also a possibility that the changes observed are due to conditions within the clusters as in cancer, the centre of tumours often have necrotic conditions due to lack of access to nutrients [288].

For the purpose of immunotherapy, the culture dish surface appears to depend on what stimuli used as we saw different effects of surface adhesion with different stimulation conditions. What simulates in vivo conditions better is also questionable as immature DC reside in tissue awaiting stimuli, whereas mature DC loses anchoring properties to local tissue and migrate towards lymph nodes. Therefore physiologically speaking during generation, monocytes should technically be in non-adherent culture dishes with fluidic movement to simulate blood flow and switched to adherent culture dishes once generated into immature moDC, but this is logistically not very feasible. The overall message from our study in paper II is therefore to always provide information

regarding culture dish used and be mindful about data interpretation depending on culture dish used.

5.2.3 Challenges with studying cells from cancer patients

It is a well-known fact that cancer patients often have altered immune systems compared to healthy individuals [289]. As we found OK432 cocktail matured moDC to have properties that appeared beneficial for immunotherapy, we wanted to investigate whether moDC from prostate cancer patients reacted similar to healthy controls with the same treatment. There were however notable challenges due to various factors. Firstly, moDC for the purpose of immunotherapy requires a certified GMO grade lab for generation which is not available at our location, therefore patient PBMCs had to be shipped to Oslo for generation and shipped back. The cells were cryopreserved in liquid nitrogen, but this adds a factor which we did not have in our previous OK432 matured moDC studies. Additionally, considerable amount of apoptosis was observed in the cells once thawed and it is impossible to determine what role logistics played as no healthy control samples were sent parallel with the prostate cancer patient samples.

As generation of GMO grade moDC for therapeutic purposes was very expensive, there were limitations to how many samples we could obtain, and combined with the considerable cell loss after thawing, meant that there were restrictions on how many samples could be run (paper III). Our results, however, showed that moDCs from prostate cancer patients showed fairly similar mature phenotype regardless whether they were stimulated by a maturation cocktail or not, suggesting processes during generation of immature moDC or aftercare resulted in a mature phenotype. It is known that cryopreservation can activate immature moDC [263], but choices were limited without a local GMP grade lab. Ideally there should be no cryopreservation or delay between collection of patients PBMC and generation of moDC for therapeutic purposes, but logistically this is not currently possible.

Regardless of the lack of differences observed in the moDC phenotype of prostate cancer patients, we observed high expression of checkpoint molecules PD-L1 and PD-

L2. The T-cells stimulated also showed high expression of PD-1 on the majority of CD4+ and CD8+ cells, suggesting that immunotherapy on these patients likely benefit from checkpoint inhibitors. Whether this is a property of prostate cancer patients, however, is not answerable in our study as we do not have healthy controls included in the logistics portion of the study. However, our study is in line with some observations by another study that show increased PD-1 expression by T-cells in prostate cancer patients [290].

The expansion of CD25+ memory cells by OK432 cocktail matured moDC in prostate cancer patients is interesting, but what it indicates is somewhat questionable. As OK432 contains components of Streptococcus pyogenes, a very common bacteria, it should come as no surprise that memory cells are activated if moDC present these antigens. We did use PPD as a recall antigen and PPD antigen specific IFN-γ response was observed regardless of moDC treatment, but as only 3 patients were included with varying results it is hard to draw any conclusions.

6. Conclusions

The challenges and results from the three studies included in this thesis highlight the complex nature of moDC generation and how easily monocytes are affected by various factors that are often overlooked. Future studies on applicability of moDC for immunotherapy should take into consideration of those factors when developing their protocols.

OK432 matured moDCs show promising aspects for immunotherapy due to phenotype with high expression of HLA-DR, CD83, CD80, CD86 and most importantly, the lack of CCR7 expression was remedied with the addition of PGE_2 in the cocktail with limited immunosuppressive effect. IL-12p70 secretion by moDC was further increased with the addition of CL097. The functionality of the CCR7 expression was confirmed by transwell assay. T-cell response could be induced by OK432 cocktail matured moDC and IL-12p70 secretion by OK432 cocktail matured moDC was comparable to many other maturation cocktails.

Surface adhesion properties of culture dishes highly impact the phenotype and cytokine profile of generated moDC in many ways that vary depending on culture condition. It is highly suggested future studies take these considerations into account and always include details about culture conditions apart from medium and stimulants.

Logistical considerations should be considered when designing moDC studies. Patient materials might not be as readily available, and cryopreservation needs to be evaluated for feasibility regarding moDC for immunotherapy. T-cells from metastatic prostate cancer patients included in our study were capable of being stimulated by autologous moDC.

7. Future perspectives

While we have done many preliminary studies on the potential of OK432 as a maturation stimulus for moDC, more remains to be done regarding their feasibility for immunotherapy. The results from paper I revealed some interesting features that should be taken into consideration. Notably the cytokine profile of OK432 cocktail matured moDC having reduced IL-8 should be investigated whether it is functional or not. It might be interesting to conduct a migration assay to check autologous neutrophil chemotaxis towards these moDCs compared to those matured by the Jonuleit cocktail.

The findings regarding PGE_2 are particularly interesting as while its effect on chemotaxis has been confirmed by other studies to be relevant, limited studies have been done on the effect of different concentrations of PGE_2 on migratory capability of moDC [201, 291].

Apart from the significant findings in paper I, the number of samples are on the low side, so more repetitions are always welcome to clarify some of the results. Additionally, as we have revealed in paper II, different stimuli affect cells differently depending on cell culture surface. Therefore, it might be interesting to investigate OK432 cocktail matured moDC compared with Jonuleit cocktail matured moDC in different culture surface conditions as that was observed regarding Jonuleit cocktail and LPS stimulated moDC.

The findings of paper II also suggest that reconsiderations need to be highlighted among immunologists regarding the culture containers used as clearly monocytes react differently to surface adhesion than many other cells. Additionally, it would be interesting to see whether these changes are concentration dependent as clearly the cells in non-adherent surface containers formed homotypic clusters and the density of the clustering effect itself might contribute to the changes observed. As we revealed CD18 to be the main contributor to the clustering effect, what role CD18 plays in moDC generation should also be investigated as well as the role of CD11a and CD11b

as potential competitive inhibitors. It would also be interesting to see if blocking CD18 affects cells generated in standard cell culture dish surfaces.

Finally, the study conducted in paper III did not meet its goal of determining whether moDC generated from metastatic prostate cancer patients could benefit from OK432 cocktail stimulation because control for the logistics part of the study was not included. This makes it impossible to determine if changes observed are due to processes such as cryopreservation, handling and delay or properties associated with the disease. Therefore, future studies should ideally avoid factors that might influence the results and always include controls undergoing the same treatment.

What did differ in paper III, however, is that there appears to be a clear expansion of CD25+ memory T cells by OK432 cocktail stimulated moDC. While we did use PPD as recall antigen, it remains to be seen if OK432 stimulated moDC also expand CD25+ cells in the absence of recall antigen and could be investigated in the future.

The final key message of our findings is the necessity to include checkpoint inhibitors when using moDC for immunotherapy. High expression of checkpoint molecules was observed in both paper II and paper III regardless of moDC treatment, therefore it is unlikely to achieve notable immunogenic stimuli without inclusion of checkpoint inhibitors.

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Papers I-III

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The Culture Dish Surface Influences the Phenotype and Cytokine Production of Human Monocyte-Derived Dendritic Cells

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Monocyte-derived dendritic cells (moDC) are an important scientific and clinical source of functional dendritic cells (DC). However, the optimization of the generation process has to date mainly been limited to the variation of soluble factors. In this study, we investigated the impact of the cell culture dish surface on phenotype and cytokine profile. We compared a standard cell culture dish to a non-adherent culture dish for two immunogenic maturation conditions, two tolerogenic conditions, and an unstimulated control. Phenotype, cytokine profile and T cell stimulatory capacity were determined after a 3-day culture. Light microscopy revealed an increase in homotypic cluster formation correlated with the use of non-adherent surfaces, which could be reduced by using blocking antibodies against CD18. All surface markers analyzed showed moderate to strong differences depending on the culture dish surface, including significantly decreased expression of key maturation markers such as CD80, CD86, and CCR7 as well as PD-L1 on cells stimulated with the Jonuleit cytokine cocktail cultured on a non-adherent surface. Significant differences in the secretion of many cytokines were observed, especially for cells stimulated with LPS, with over 10-fold decreased secretion of IL-10, IL12-p40, and TNF-α from the cells cultured on the non-adherent surface. All immunogenic moDC populations showed similar capacity to induce antigen-specific T cells. These results provide evidence that the DC phenotype depends on the surface used during moDC generation. This has important implications for the optimization of DC-based immunotherapy development and underlines that the local surrounding can interfere with the final DC population beyond the soluble factors.

Keywords: homotypic clusters, monocytes, monocyte-derived dendritic cells, immunogen, tolerogen, adhesion, non-adherent culture plate, cytokines

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INTRODUCTION

Dendritic cells (DC), positioned between the innate and adaptive immune system, play a central role in a great variety of immunological settings. They play an important role for the pathogenesis of many diseases, and are increasingly also under investigation as a clinical tool to treat a great diversity of different challenging conditions, ranging from cancer to autoimmunity (1). The role of DC in medicine has already been highlighted by their discoverer Ralph M. Steinman (1, 2),

ranging from infectious diseases over autoimmunity to cancer (3–5). However, the complexity of the pathogenic settings in the immune system and the diversity of DC subtypes are contributing to an enduring challenge to understand and apply DC biology. Many obstacles have to be overcome on the way to clinics, but the understanding of the *in vitro* system for the development of DC applications is of special importance. As DC are a central sensing unit collecting all information before a possible activation of the adaptive immune system, it is not surprising that the culturing environment can have a great impact on the cellular phenotype and thus on the induced immune response. Commonly, blood monocytes are the major source of cells to generate DC *ex vivo*, mainly because they are readily accessible. Monocyte-derived DC (moDC) are then generated by use of conditioning soluble factors, usually a combination of GM-CSF and IL-4, to induce the DC program in monocytes, followed by a maturation cocktail that mimics an *in vivo* maturation condition leading to the desired phenotype. For example, one of the commonly used maturation cocktails for immunogenic DC is one that imitates an inflammatory situation in the skin (referred to as "Jonuleit cocktail"), containing IL-1β, IL-6, TNF, and prostaglandin E2 (PGE_2) (6). Moreover, serum-free formulations are recommended to ensure reproducibility and achieve compliance with clinical requirements (7). However, only the impact of soluble factors is commonly considered, the adhesional culture properties are hugely ignored in most *in vitro* protocols. If mentioned at all, standard cell culture plates are recommended. Alone the *in vivo* regulation of DC adhesion upon maturation (8, 9) gives an indication that adhesional signaling might be of importance in a potentially more diverse way than can be expected from an unspecific surface of a plastic cell culture dish. In connection with the culturing conditions, we observed an early increase in DC markers on immature DC when cultured on non-adherent surfaces compared to standard cell culture dishes (10). In the same study, we observed an increase in homotypic clustering of the cells on non-adherent surfaces compared to cells on standard cell culture plates. Thus, the choice of the culture dish can potentially have a significant impact on the DC phenotype and function by either supporting the early, integrin-mediated adhesion followed by low homotypic clustering, or by reducing culture dish interactions leading to an increase in clustering and thus cluster-mediated cell-cell interactions.

The aim of the present study was to investigate the effect of the culture dish surface on the phenotype and the cytokine production of differentially stimulated immunogenic and tolerogenic moDC populations. We found that both phenotype and cytokine secretion are modulated in a treatment-dependent manner. Moreover, using blocking antibodies, we determined CD18 as the most important molecule for the homotypic cluster formation.

MATERIALS AND METHODS

Dendritic Cell Generation

Freshly drawn peripheral blood was collected from 19 healthy volunteers into BD Vacutainer ACD-A 10 ml citrate tubes (BD, Franklin Lakes, USA). Informed consent was obtained from all donors. The study was approved by the regional ethical committee Western Norway (REK Vest; #2009/686). The age of the donors was ranging from 23 to 67 years. Monocytes were isolated as described previously (10). In short, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep (Axis- Shield, Oslo, Norway). The PBMC were washed twice and centrifuged at 220 g for 8 min at 4°C, respectively, in order to further increase the leukocyte to platelet ratio. Monocytes were further isolated using the Monocyte Isolation Kit II (Miltenyi Biotec Norden AB, Lund, Sweden). In four experiments, additional anti-CD61 microbeads (Miltenyi Biotec Norden AB, Lund, Sweden) were added to reduce residual platelet numbers. The final untouched monocyte fraction was washed, counted on a CASY cell counter and resuspended in serum free CellGro DC medium (CellGenix GmbH, Freiburg, Germany). During culture, Nunclon Δ 6-well plates and Nunc HydroCell 6-well plates were used in comparison as representative standard culture dish and non-adherent culture dish, respectively (Thermo Fisher Scientific, Waltham, USA). For the MLR and the blocking antibody experiments, the non-adherent culture dish was changed to Nunc Sphera (Thermo Fisher Scientific, Waltham, USA), the newer line of non-adherent culture plates, due to discontinuation of the HydroCell series. 0.75×10^6 monocytes/ml CellGro DC medium were plated per surface and per maturation stimulus, supplemented with IL-4 (20 ng/ml) and GM-CSF (100 ng/ml) (ImmunoTools, Friesoythe, Germany). IL-4 and GM-CSF were replenished after 2 days, 24 h before cell harvesting.

For 5 of the donors, blocking experiments were performed. For the other 14 donors, five different DC populations were generated for each surface, two of them immunogenic (LPS and Jonuleit cytokine cocktail, respectively), two tolerogenic (Dex/VD3 and IL-10, respectively) and one control sample without additional stimulus. LPS (100 ng/ml; Sigma-Aldrich, Taufkirchen, Germany) and the Jonuleit cytokine cocktail [10 ng/ml of IL-1β, 10 ng/ml of TNF, 1,000 U/ml of IL-6; all ImmunoTools, Friesoythe, Germany, and 1 μg/ml of prostaglandin E2 (PGE_2); SigmaAldrich, Taufkirchen, Germany] were added 24 h before harvesting, respectively. For the generation of DexVD3 DC, 1 μM of dexamethasone (Dex; Sigma- Aldrich, Taufkirchen, Germany) was added at the start of culture, and replenished 24 h before harvesting together with 1 nM of 1α, 25-Dihydroxyvitamin D3 (VD3) (Enzo Life Sciences, Farmingdale, NY). For the generation of IL-10 DC, IL-10 (10 ng/ml) was added at culture start and replenished 24 h before harvesting (Miltenyi Biotec Norden AB, Lund, Sweden). As DMSO (Sigma-Aldrich, Taufkirchen, Germany) was used as a solvent for Dex and VD3, a corresponding amount of DMSO was added to the control samples (DMSO iDC). All cells were harvested after 3 days in culture. Cell-free supernatants were stored at −20°C for later cytokine detection. The remaining cells were washed off the surfaces with PBS (without magnesium and calcium; Lonza, Verviers, Belgium) containing 2 mM EDTA (Sigma- Aldrich, Taufkirchen, Germany). The viability of the generated DC population for each condition was determined by annexin-V and 7-AAD staining using the Annexin V Apoptosis Detection Kit (eFluor

450) from eBioscience (AH Diagnostics, Oslo, Norway) and a LSRFortessa cell analyzer (BD, Franklin Lakes, USA) located at the Core facility for Flow cytometry, Dept. of Clinical Science, University of Bergen.

Blocking Antibodies

In some experiments, blocking antibodies against CD11a (clone HI111), CD11b (clone ICRF44), CD11c (clone 3.9), CD18 (clone TS1/18; all 10 µg/ml; BioLegend, San Diego, CA, USA), or E-cadherin (clone HECD-1; 10 µg/ml; Invitrogen/Thermo Fisher, Waltham, MA, USA), all low endotoxin, azide free, were added in the growth medium for 3 days during moDC generation. As a control, the moDC were cultured with mouse IgG1 (BioRad, Hercules, CA, USA) supplemented in the growth medium in the same concentrations as the blocking antibodies. A cell population with no additional antibodies served as a negative control. After 3 days of culture, the morphology of the generated moDC populations was analyzed by light microscopy using a Cytation 5 Cell imaging-reader (BioTek instruments, Winooski, VT, USA), before the cells were harvested for phenotyping.

Immunostaining

The phenotype of the generated moDC populations was analyzed using the surface markers shown in **Table 1**. Cells were pre-incubated for 5 min using 2 µl of FcR blocking reagent (Miltenyi Biotec Norden AB, Lund, Sweden) per up to 10^6 cells in 150 µl cold PBS containing 0.5 % bovine serum albumin (BSA; Sigma-Aldrich, Taufkirchen, Germany), followed by an incubation with the titrated amounts of antibodies for 15 min in the same buffer. The flow cytometry analysis was performed at the Core facility for Flow cytometry, Dept. of Clinical Science, University of Bergen, using a BD LSRFortessa cell analyzer.

TABLE 1 | Mouse monoclonal anti-human antibodies with fluorophores and clone IDs used for flow cytometry analysis.

Antigen	Fluorophore	Clone
CD14	FITC	18D11
CD1a	PE	HI149
CD38	PerCP-Cy5.5	HIT2
CD83	PE-CF594	HB15e
CD40	PE-Cy7	5C3
CD86	Alexa Fluor 647	IT2.2
CD80	Brilliant violet 605	2D10
HLA-DR	Horizon V500	G46-6
CD197 (CCR7)	Brilliant violet 421	G043H7
CD18	FITC	TS1/18
CD11c	PerCP-Cy5.5	3.9
HLA-A,B,C	APC-Cy7	W6/32
CD274 (PD-L1)	PE-Cy7	MIH1
CD273 (PD-L2)	Alexa Fluor 647	MIH14
CD11b	Brilliant violet 421	ICRF44

Cytokine Detection

For the detection of cytokines in the cell medium after DC generation, we used a magnetic microbead based 25-plex human cytokine kit for the Luminex platform (Invitrogen Corp., Carlsbad, USA). The cytokines measured were IL-1β, IL-10, IFN-α, IL-6, IL-12, RANTES (CCL5), Eotaxin (CCL11), IL-13, IL-15, IL-17, MIP-1α (CCL3), GM-CSF, MIP-1β (CCL4), MCP-1 (CCL2), IL-5, IFN-γ, TNF-α, IL1RA, IL-2, IL-7, IP-10 (CXCL10), IL-2R, MIG (CXCL9), IL-4, and IL-8. All supernatants were thawed and analyzed simultaneously. Measured median fluorescence intensity (MFI) values below the standard-curve were set to the detection limit and cytokines with MFI-values above the standard-curve were approximated by extrapolating linearly.

Mixed Leukocyte Reaction (MLR)

In order to analyze the T cell stimulatory capacity of the generated moDC populations, we performed allogeneic mixed leukocyte reactions (MLR) as described previously (11). 5×10^4 moDC were co-cultured with 2×10^5 monocyte depleted PBMC stained with CFDA-SE (Vybrant CFDA-SE Cell Tracer Kit, Thermo Fisher Scientific, Waltham, USA) for 5–7 days in X-Vivo20 medium supplemented with IL-7 (10 ng/ml) and IL-2 (50 U/ml; both ImmunoTools, Friesoythe, Germany). At least 30,000 events were collected on a BD Accuri C6 instrument at the Core facility for Flow cytometry, University of Bergen.

IFN-γ Secretion Assay

An IFN-γ secretion assay (Miltenyi Biotec, catalog number 130-054-202) was utilized to analyze the capacity of the generated DC populations to induce antigen specific T cell responses as described previously (11). In short, 2.5×10^6 autologous monocyte-depleted PBMC were co-cultured with 5×10^5 PPD-loaded DC populations for 7 days in X-Vivo20 medium supplemented with IL-7 (10 ng/ml) and IL-2 (50 U/ml). The IFN-γ secretion assay was performed according to the manufacturer's manual. As stimulators, PPD-loaded DC stimulated with LPS were used, and unloaded LPS-DC served as negative control. 2×10^5 DC were co-cultured with 8×10^5 induced monocyte-depleted PBMC for 16 h. Staphylococal enterotoxin B from Staphylococcus aureus (SEB; 1 µg/ml; Sigma-Aldrich) was added as positive control. Prior acquisition on a BD LSR Fortessa, the cells were stained with anti-CD4 FITC (M-T466, Miltenyi Biotec) and anti-CD8-APC (RPA-T8, Biolegend), as well as 7-AAD (ebioscience). A minimum of 2×10^5 events were collected in the Lymphocyte gate. FlowJo was used to analyze the data, and % IFN producing cells were calculated according to the following formula:

$$\%\ \text{IFN}\gamma\ \text{producing cells among CD4}^+ = \frac{\#\ of\ \text{IFN}\gamma^+\ \text{CD4}^+\ \text{cells in the sample}}{\#\ of\ \text{total CD4}^+\ \text{cells in the sample}} \times 100$$

IFN-γ producing CD8+ T cells were calculated accordingly. The % IFN-γ producing cells from DC without PPD was subtracted from the values with PPD.

Statistical Analysis

The statistical analyses were performed with Graph-Pad Prism (v5.02). Statistical significance was determined by comparing the cells of the two different surfaces for each treatment using a 2way ANOVA test in combination with the Bonferroni post-test or Dunn's post-test, significance criterion <0.05. Significance values in figures are given in grades $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Median values for each surface and treatment are marked with a line. Measured values are given as the mean ± the standard deviation (SD) if not stated otherwise.

RESULTS

The Lack of Surface Adherence Leads to Increased Homotypic Cluster Formation That Can Be Reduced by Blocking CD18

Using light microscopy, we observed an increase in cell cluster formation on the non-adherent culture dish in comparison to the standard cell culture dish for all moDC populations (**Figure 1**, **Figure S1**). Considerably smaller clusters were also observed on the standard cell culture dish, especially for the immunogenic stimulation conditions with LPS or the Jonuleit cocktail (**Figure S1**). Viability was not influenced by the different surfaces (data not shown). In order to determine the molecules responsible for this clustering behavior, we added various blocking antibodies to the culture. Blocking CD18 reduced clustering on the non-adherent plates (**Figure S2**), while blocking CD11a and CD11b appeared to promote homotypic clustering, independent of the surface used. Blocking CD11c and E-cadherin had inconsistent results.

Culture Dish Adherence Influences Phenotype and Cytokine Production of moDC

We further investigated phenotypic differences depending on the treatment and surface conditions by analyzing expression levels of 15 different surface markers using flow cytometry (**Figure 2**). The expression of all cell surface molecules was influenced by the culture dish used. Interestingly, the use of a non-adherent culture dish decreased expression of PD-L1 on cells treated with the Jonuleit cocktail, while the other PD-ligand, PD-L2 (CD273), was highly expressed.

Cytokine production varied depending on the treatment and the surface used (**Figure 3**). In general, LPS-DC had the highest production of most cytokines. Regarding the influence of the culture dish surface, most cytokines were overall secreted at lower levels on non-adherent surface. Interestingly, the levels of exogenously added cytokines GM-CSF and IL-4 varied a lot. GM-CSF levels were higher on the standard cell culture dish than on the non-adherent surface, while IL-4 levels were lower. While the cytokine profile of the DC cultured in tolerogenic conditions as well as control showed limited differences in cytokine profile regardless of culture dish surface, the LPS stimulated DCs had significant profile differences. Interestingly, IL-10 and TNF-α secretion was increased in all LPS stimulated DCs on standard cell culture dish compared to non-adherent surface, while the opposite was true for IL-15 and MIG.

Blocking of adhesion molecules resulted in little differences in surface expression of most markers analyzed (data no shown).

Additional Platelet Reduction During Monocyte Isolation Has No Distinct Effect on Phyenotype and Cytokine Production

In order to investigate the possibility that isolation impurities might have an impact on the clustering, phenotype, and cytokine production, four of the monocyte isolation procedures were

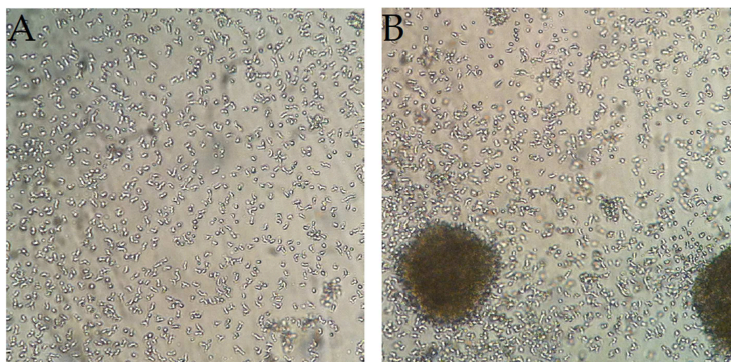

FIGURE 1 | Homotypic cell clusters form on the non-adherent surface but less on the standard culture dish. Representative microscopy pictures of iDC (DMSO) at the end of the 3-day culture on **(A)** a standard cell culture dish and **(B)** a non-adherent culture dish. The increase in cell cluster formation on non-adherent dishes relative to standard dishes could be observed for all treatments ($n = 8$).

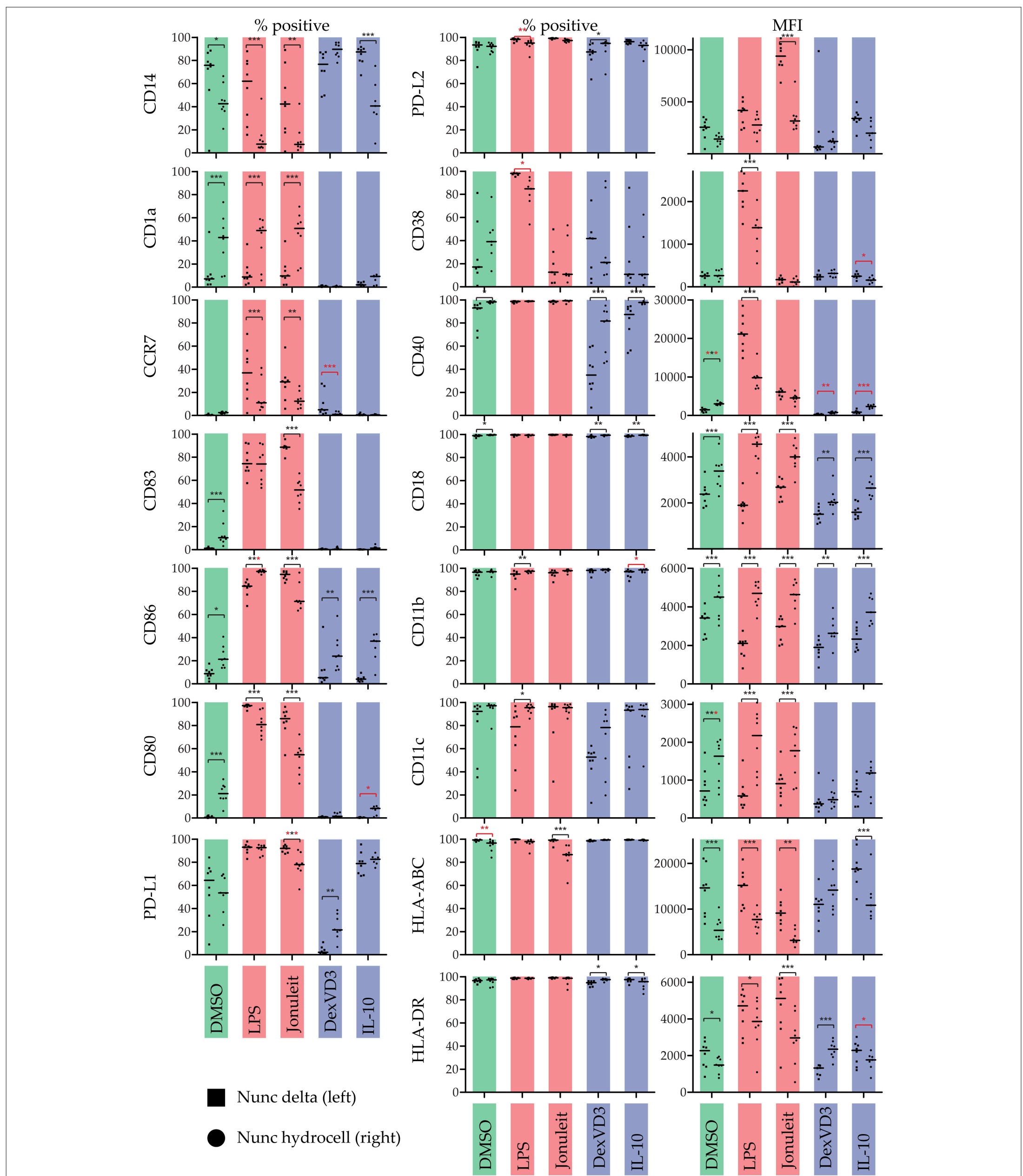

FIGURE 2 | Influence of the culture dish on the phenotype of differentially stimulated moDC. The phenotype was analyzed by flow cytometry using the indicated surface molecules. % positive cells or median fluorescence intensity (MFI) are shown. Color code: Control (DMSO)—green, immunogenic DC populations (LPS & Jonuleit cytokine cocktail)—red, tolerogenic DC populations (Dexamethasone with vitamin D3 & IL-10)—blue. Squares **(left)**: standard culture dish; circles **(right)**: non-adherent culture dish. The median is marked with a line. Significance values are given in grades $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Black significance grades are the result of a collective 2 way ANOVA testing for all treatments, red grades are from separate tests for the iDC/tolDC and immunogen subgroups ($n = 8$).

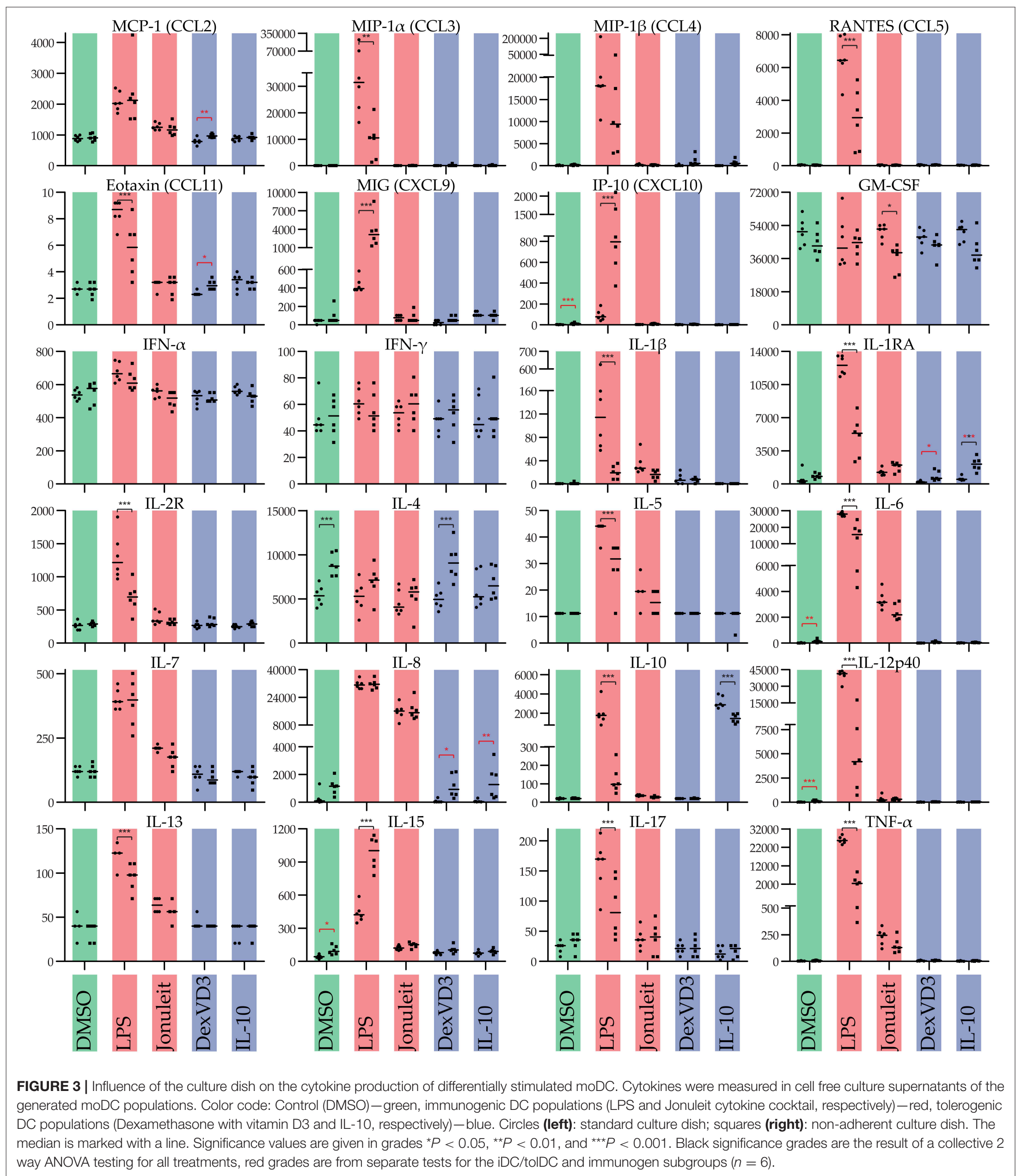

FIGURE 3 | Influence of the culture dish on the cytokine production of differentially stimulated moDC. Cytokines were measured in cell free culture supernatants of the generated moDC populations. Color code: Control (DMSO)—green, immunogenic DC populations (LPS and Jonuleit cytokine cocktail, respectively)—red, tolerogenic DC populations (Dexamethasone with vitamin D3 and IL-10, respectively)—blue. Circles **(left)**: standard culture dish; squares **(right)**: non-adherent culture dish. The median is marked with a line. Significance values are given in grades $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Black significance grades are the result of a collective 2 way ANOVA testing for all treatments, red grades are from separate tests for the iDC/tolDC and immunogen subgroups ($n = 6$).

performed using additional anti-CD61 beads to remove platelets. While the monocyte purity without the use of anti-CD61 beads was >85 %, it increased to >95 % when additional anti-CD61 beads were used (**Figure S3**), confirming that platelets were the main impurity. However, both the phenotype of the generated DC populations and their produced cytokines did not show any clear differences between the preparations with and without residual platelets (data not shown).

The Cell Culture Surface Does Not Influence the T Cell Stimulatory Capacity of moDC

Lastly, we analyzed the T cell stimulatory capacity of the generated moDC populations. Using allogeneic MLR, no obvious differences were observed between the different surfaces, regardless of the DC population used as stimulator (**Figure S4**). As expected, the co-culture with the immunogenic DC populations (LPS and Jonuleit-cocktail stimulated cells) resulted in higher proliferation compared to the immature control DC. The proliferation of monocyte-depleted PBMC co-cultured with the tolerogenic moDC populations was even less than with immature DC. We further analyzed the immunogenic moDC populations in an autologous setting. Both LPS- and Jonuleit-cocktail stimulated cells were able to induce antigen specific autologous CD4+ and CD8+ T cells, with slightly higher numbers of IFN-γ producing T cells upon using LPS-stimulated DC, independent of the culture dish used (**Figure 4**).

DISCUSSION

In this study, we analyzed the influence of the cell culture surface on differentially stimulated moDC. The formation of cell clusters was increased for all cells cultured on the non-adherent culture dishes. Cell clusters on the standard culture dish were in comparison very small and only forming after immunogenic stimulation, suggesting a different clustering mechanism. The phenotype was in many cases significantly modulated depending on the cell culture dish. A total of 17 of the 25 measured cytokines were secreted at significantly different levels depending on the culture dish by moDC stimulated with LPS. However, the T cell stimulatory capacity was not influenced by the culture surface.

As immature DC are known to have tolerogenic functions (12), and based on the similarities in phenotype and cytokine production between the iDC and tolDC in our study, iDC/tolDC will be discussed collectively. In contrast, due to the distinct differences observed between LPS-DC and Jonuleit-DC, they will be discussed separately.

Immature DC and Tolerogenic DC

The typical monocyte/macrophage marker CD14 was expressed at high levels in all iDC/tolDC populations on the standard culture dish, but at lower levels in the non-adherent culture dish, with the exception of DexVD3 DC. This is in line with a previous study showing that culturing monocytes in suspension rather than adherent conditions leads to a rapid reduction in CD14 (13). Thus, using CD14 as a marker for successful DC generation when comparing adherent with suspension protocols might not be the best choice. Nevertheless, the prevention of the suggested internalization of CD14 by DexVD3 treatment is interesting and should be investigated further.

The lack of DC maturation marker CD83 on all iDC/tolDC populations confirmed their immature state. Interestingly, iDC cultured on non-adherent surfaces showed a slight but statistically significant increase in CD1a, CD83, and CD80 expression, indicating that the formation of clusters might lead to a "spontaneous" DC maturation. This hypothesis is further strengthened by our observation that blocking CD11b, resulting in increased homotypic clustering on the non-adherent surface, also led to increased expression of costimulatory molecules and MHC. This effect was not observed in the other tolDC populations. However, further studies will have to investigate the effect of the clustering on the tolerogenic function of the different moDC populations *in vitro* and *in vivo*.

LPS Matured DC

LPS-stimulated moDC on standard cell culture dishes were, as expected, $CD83^{+}$ $CD40^{high}$ $CD86^{high}$ $CD80^{+}$ MHC-II^{high} and increasingly $CCR7^{+}$. Using a non-adherent culture dish leading

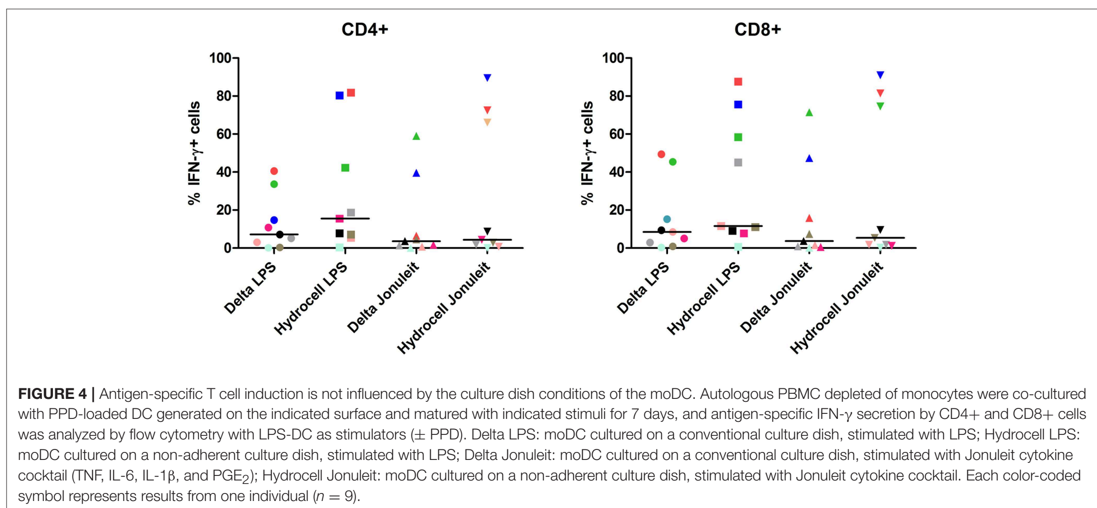

FIGURE 4 | Antigen-specific T cell induction is not influenced by the culture dish conditions of the moDC. Autologous PBMC depleted of monocytes were co-cultured with PPD-loaded DC generated on the indicated surface and matured with indicated stimuli for 7 days, and antigen-specific IFN-γ secretion by CD4+ and CD8+ cells was analyzed by flow cytometry with LPS-DC as stimulators (± PPD). Delta LPS: moDC cultured on a conventional culture dish, stimulated with LPS; Hydrocell LPS: moDC cultured on a non-adherent culture dish, stimulated with LPS; Delta Jonuleit: moDC cultured on a conventional culture dish, stimulated with Jonuleit cytokine cocktail (TNF, IL-6, IL-1β, and PGE_2); Hydrocell Jonuleit: moDC cultured on a non-adherent culture dish, stimulated with Jonuleit cytokine cocktail. Each color-coded symbol represents results from one individual ($n = 9$).

to the formation of clusters had a great impact on the phenotype and cytokine secretion of the LPS-DC. Interestingly, based on the CD83 expression, the percentage of mature DC did not change, indicating that the observed changes reflect a change of polarity but not maturation. Especially interesting was the decrease in secretion of CC chemokine family members CCL3, CCL4, and CCL5, which are all ligands of CCR1 and CCR5. These chemokines attract mainly cells of the innate immune system like granulocytes (14), monocytes/macrophages (15), NK cells (16), mast cells (17), or immature dendritic cells (18) but also $CD8^+$ T cells (16, 19). Interestingly, IL-8, another chemokine of the innate immune system attracting neutrophils (20), was not modulated and secreted in equally high amounts. Strikingly, the chemokines CXCL9 and CXCL10, both ERL-negative ligands of CXCR3, were significantly more secreted. Especially CXCL10 had been nearly absent on LPS-DC on the standard culture dish, but was secreted by moDC on the non-adherent culture dish. Both chemokines of the CXC family have been associated with supporting T helper 1 differentiation *in vivo* (21) but their receptor CXCR3 appears also to be essential during wound healing (22). CXCL9 and CXCL10 have also been reported to recruit activated IFN-γ expressing NK cells, $CD8^+$ T cells and $CD4^+$ T cells (23). The anti-inflammatory cytokines IL-1RA, IL-2R, and IL10 were also secreted at lower levels. Also IL-6 secretion was reduced considerably. IL-6 has been associated with maintaining immature DC (24) but is also linked to T helper 17 polarity (25). While IL-17 was not secreted as much, it also was reduced upon use of non-adherent culture dishes. Interestingly, IL-15 secretion was significantly increased by LPS-DC cultured on a non-adherent cell culture dish. IL-15 is a cytokine secreted mainly by monocytes/macrophages and dendritic cells (26, 27) and has gained a special interest as it is required for the differentiation of NK cells, effector $CD8^+$ T cells and memory $CD8^+$ T cells (26). IL-15 is also involved in antiviral immunity by formation of IL-15-IL-15Rα complexes able to induce IFN-γ mediated responses independent of type I IFN (23).

Taken together, LPS-DC shift from a CC chemokine response to a CXC chemokine response when modifying the culture dish from a standard cell culturing condition to a non-adherent cell culture dish. The high CD40 expression and high secretion of chemoattractants by cells cultured on the standard cell culture dish might be an example of an "all out" immune response, which is only kept under control by anti- inflammatory cytokines like IL-1RA, IL2R, and IL-10. The moderation of many of these factors on the non-adherent culture dish combined with an upregulation of CD86, IL-15, and the T cell chemoattractants CXCL9 and CXCL10 suggest a rather directed response, probably of a T helper 1 direction. Further *in vitro* and *in vivo* studies are needed to analyze the functional impact of the non-adherent culture dish during LPS- DC generation.

DC Matured With Jonuleit Cytokine Cocktail

Interestingly, Jonuleit cytokine cocktail stimulated DC behaved very differently than LPS-DC. CD83 expression was significantly reduced when using a non-adherent cell culture dish, indicating less mature DC. Thus, most of the other observed changes in phenotype markers can be explained with the lower amount of mature DC. Surprisingly, there was no significant difference in the cytokine production except for GM-CSF between Jonuleit-DC cultured on standard cell culture and non-adherent cell culture dishes. In comparison to iDC/tolDC, most cytokines were not secreted significantly different.

Taken together, the impact of the change in cell culture surface seems more predictable for the Jonuleit DC. The use of a non-adherent culture dish will probably not induce a different polarity but would reduce the number of immunostimulatory DC. While this is interesting with regard to *in vivo* mechanisms controlling inflammation, a non-adherent cell culture surface might not be the best choice when aiming at high numbers of immunogenic DC to be used for immunotherapy, even though the T cell induction capacity was not impaired. However, we here used a recall antigen (PPD), and did not analyze the capacity to induce naive T cells, meaning the effect of culture dish adherence on DCs ability to process and present novel antigens remains to be explored. This suggests that future studies on the matter should consider utilizing another antigen with no prior memory presence and isolate naïve T-cells with negative selection prior to T cell induction capacity analysis. Another possibility to address this question would be to test the T cell induction capacity of DC on naïve T-cells from transgenic mice with known antigen specificity. In our experiments, the cytokines contained in the Jonuleit cocktail have rather broad inflammatory functions and give more of a "danger" stimulus, while LPS binds to TLR4, an innate pattern recognition receptor with specific function and predefined certainty of pathogenic recognition. The close contact with "self" in the clusters might rather calm down the unspecific stimulation. The LPS-DC on the other hand can rather be sure of an immediate danger and can than only be modulated to change polarity instead of remaining dormant. This gives an indication of when the change to the non-adherent culture conditions might lead to new subtypes of DC.

Possible Mechanisms for the Induction of the Observed Culture Dish Dependent Differences

Based on microscopy observations, the main consequence of using a standard cell culture dish during moDC generation is an early adherence phase, while moDC cultured on a non-adherent surface form homotypic clusters. Usually, adherent monocytes will detach during the first day in DC culture conditions (10, 28). After the detachment, the characteristics of the cell culture dish should not influence the floating cells any longer. However, the early adhesion prevents the floating cells to cluster afterwards. It is unclear if the early adhesion is only important to control clustering or if the early adhesion alone already triggers signaling pathways which will lead to the phenotype differences, independent of the following clustering. Intriguingly, all three integrins analyzed, CD11b, CD11c, and CD18, were significantly higher expressed on moDC cultured on the non-adherent culture dishes (**Figure 2**). However, it is unclear if this is connected to the mechanism inducing the phenotype changes. Integrin-binding has been shown to induce DC maturation (29), and there is evidence suggesting that the conversion of monocytes to DC can be supported by specific integrin-binding (30).

Based on the high expression levels, we chose to utilize blocking antibodies against CD11a, CD11b, CD11c, CD18, and E-cadherin. It has been shown previously that CD11d/CD18 and CD11c/CD18 play a role in myeloid cell adhesion and spreading (31, 32). Surprisingly, only anti-CD18 consistently reduced the homotypic cluster formation in our study, suggesting other additional molecules to be involved. In contrast, blocking CD11b led to increased clustering in all our samples. This phenomenon has previously been observed by another study on blocking CD11b on moDC that suggests CD11b to be a competitive inhibitor of other more prominent integrins, thus resulting in stronger adhesive properties of moDC when blocked (33).

Homotypic clusters did not only form more intensively on the non-adherent surface, but they also persisted over days, thus showing a totally different dynamic as the early surface adhesion on the standard adherent dish. However, as detached monocytes on the standard dish did not form homotypic clusters prior to stimulation, there might also be a different integrin regulation involved in the homotypic aggregation at that point. Homotypic clustering or aggregation of DC has been observed *in vitro* and *in vivo*, but its natural function is unknown. However, support for both maturation and antigen-transfer as possible mechanism has been observed (34). It is tempting to speculate that differently matured cell types like infiltrating monocytes, locally developing DC and resident mature DC populations might cooperate in this way, helping immature cells to mature and transfer the original antigen-information to developing migrating cells in order to stimulate the adaptive immune system without having to abandon the site of inflammation. However, the effects reported and assigned to the homotypic cluster formation might also overlap with the reduction of integrin activation or other interactions of the cells with their surroundings. Further investigations will have to distinguish between these sources of influence. Future experiments should also address other culture surface conditions such as glass or other container conditions such as culture bags.

CONCLUSIONS

The use of a non-adherent surface instead of a standard culture dish can have a great impact on the phenotype and the cytokine production of differently stimulated moDC. Further investigations will be required in order to elucidate the molecular mechanisms for the effect, but differences in the early direct surface interactions and in the frequency and amount of cell-cell interactions, influenced by homotypic cluster formation, might play a deciding role. This study proves that monocytes are crucially influenced by the near surrounding during the development into dendritic cells. This has a potential application for DC mediated immunotherapy, where the cellular phenotype is essential for the success of the treatment.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by regional ethical committee for medical and health related research (#2009/686). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS and SA: conceived of the study. AS, SA, and DY: design of the study, analyzing data, and writing the manuscript. AS, DY, SR, and YL: generating data for the study. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02352/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary file

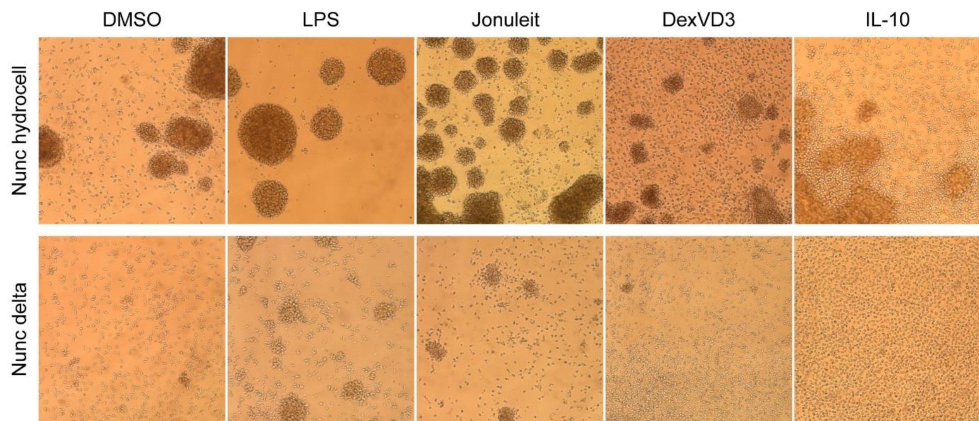

Figure S1: Homotypic cell clusters form on the non-adherent surface but less on the standard culture dish. Representative microscopy pictures of all generated DC populations at the end of the 3-day culture on a non-adherent culture dish (Nunc hydrocell) and a standard cell culture dish (Nunc delta). (n = 8)

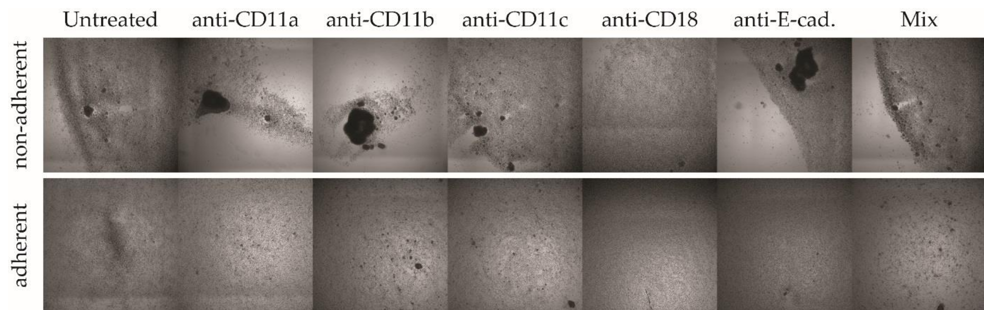

Figure S2: The effect of blocking cell adhesion molecules on homotypic cell clustering. Representative images of moDC cultured in non- adherent surface dish (upper row) and on standard surface dish (lower row) with the addition of IgG1 (untreated), anti-CD11a, anti-CD11b, anti-CD11c, anti-CD18, anti-E-Cadherin (anti-E-cad.) and a combination of all antibodies (Mix) after 3 days. Clustering on the non-adherent surface was notably reduced with the addition of anti-CD18 and increased with the addition of anti-CD11b. (n=5)

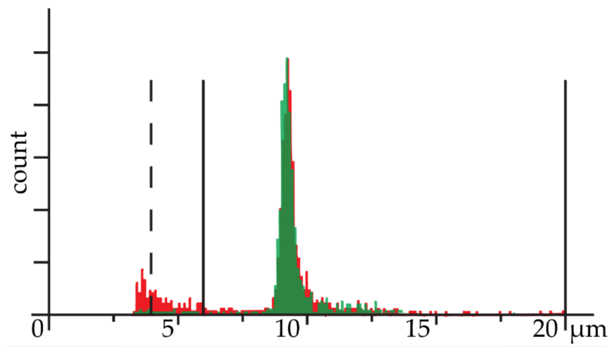

Figure S3: Improvement of the monocyte purity was achieved by further platelet removal. Representative Casy cell counter image overlay showing the initial monocyte purity with no further platelet removal (red) and with platelet removal using anti-CD61 microbeads (green). (n = 4)

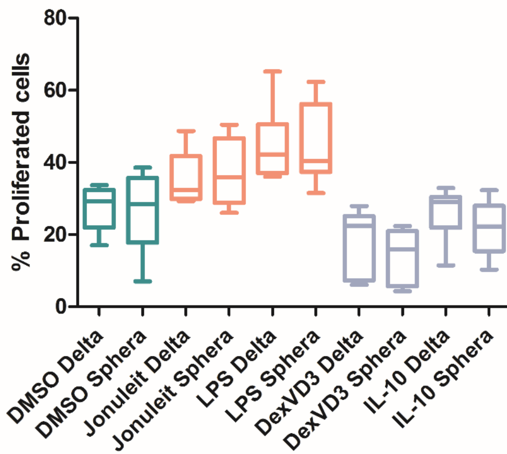

Figure S4: Mixed leukocyte reaction (MLR). Three-day moDC cultured on standard culture dish (Nunc Delta) or non-adherent culture dish (Nunc Sphera) with the addition of indicated compounds were subsequently co-cultured with allogeneic CFDA-SE stained monocyte depleted PBMC for 5-7 days. DMSO: immature moDC with DMSO control; Jonuleit: moDC stimulated with (TNF, IL-6, IL-1β and PGE2); LPS: moDC stimulated with LPS; DexVD3: moDC cultured with dexamethasone and VD3; IL-10: moDC cultured with IL-10. Percentage cell proliferation of monocyte depleted PBMC was analyzed by flow cytometry and shown as box plots with whiskers and median line. (n = 6)

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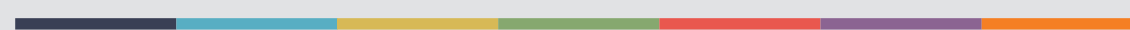

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