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Evaluation of liposomes engineered to bind toll like receptor-2 for complexation of immunostimulatory nucleic acids to target and activate antigen presenting cells
-
Evaluation von Liposomen, die Toll-like-Rezeptor 2 binden und Komplexe mit immunstimulatorischen Nukleinsäuren formen, um antigenpräsentierende Zellen zu targetieren und aktivieren

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DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

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Abstract

Dendritic cells (DCs) have become a major target for tumor therapy. Due to their significant role initiating and coordinating the human immune response, linking the innate and adaptive immune system, DCs seem to be a reasonable starting point for new approaches regarding immune therapy against cancer.

Nanocarrier-based delivery systems offer new possibilities in targeting of immune cells by facilitating the uptake of substances and the activation of the respective cells.

In this work two different nano carriers (CL419 and CL553) were tested in regard to their ability to target and activate DCs via TLR2 (and TLR7). Furthermore, different viral and synthetical oligonucleotides, serving as adjuvants, were tested, activating DC via TLR9 or STING pathway. Choosing the best performing substances of each group, complexes were formed in order to create a vaccine. The combination of some substances yielded a significant activation of DCs in-vitro. Nevertheless, in-vivo trials have not been performed yet, which will uncover the next hurdles on the path of creating an effective anti-tumor therapy. The application way of the vaccine is to be considered, taking into account the pharmacokinetics and pharmacodynamics, as well as the tumors microenvironment.

Concludingly, nano carrier based immune therapy might play an important role in fighting cancer, complementing the common therapies such as chemotherapy, radiation and check point therapy.

Zusammenfassung

Dendritische Zellen (DC) haben eine wichtige Rolle in der Tumortherapie eingenommen. Aufgrund ihrer wichtigen Funktion in der Initiierung und Koordination der Immunantwort im menschlichen Körper, wobei sie das angeborene und adaptive Immunsystem miteinander verbinden, scheinen sie ein guter Ansatzpunkt für neue Tumortherapien zu sein.
Nanocarrier basierte Systeme bieten neue Möglichkeiten Immunzellen zu targetieren, indem sie die Aufnahme und die Aktivierung der Zellen erleichtern.
In dieser Arbeit wurden zwei verschiedene Nanocarrier (CL419 und CL553) hinsichtlich ihrer Fähigkeit DC über TLR2 (und TLR7) zu targetieren und zu aktivieren getestet. Außerdem wurden verschiedene virale und synthetische Oligonukleotide, die als Adjuvantien dienen, getestet, die DC über den TLR9 oder STING pathway aktivieren.
Die Substanzen jeder Gruppe, die am besten abschnitten, wurden verwendet, um eine Vakzine zu kreieren. Durch die Kombination einiger Substanzen konnte eine signifikante Aktivierung von DC in-vitro erreicht werden. Allerdings wurden bisher noch keine in-vivo Versuche durchgeführt, durch die sich neue Hürden auf dem Weg zu einer wirksamen Tumortherapie ergeben werden. Die Art der Applikation der Vakzine muss noch überdacht werden, wobei die Pharmakodynamik und -kinetik, sowie das Microenvironment des Tumors in Betracht gezogen werden muss.
In naher Zukunft könnte die Nanotherapie ein wichtiger Faktor der Tumortherapie darstellen, indem sie die bisherigen Therapien, wie Chemotherapie, Bestrahlung und Check point Blockade, komplementier

Table of Contents

Abstract ... IV

Zusammenfassung ... V

Table of Contents ... VI

List of Abbreviations ... VIII

List of figures ... X

1. General Introduction – Immune system ... 1

1.1. Innate immune system ... *2*

1.2. Adaptive immune system ... *2*
1.2.1. APC ... 3
1.2.2. T-cells and their subsets ... 3
1.2.3. B-cells ... 5

1.3. Dendritic Cells ... *6*
1.3.1. Ontogeny of DC ... 6
1.3.2. Surface Markers ... 7
1.3.3. Toll-like receptors (TLR) ... 7
1.3.4. Interaction between DC and T-cells ... 8

2. Discussion of Literature - Cancer Immunotherapy ... 10

2.1. DC as a target for immunotherapy ... *10*

2.2. Limitations of conventional vaccines ... *11*

2.3. Advantages of nano-vaccines ... *12*

2.4. Use of different nanocarriers ... *13*
2.4.1. Passively targeting nanocarriers ... 13
2.4.2. Actively targeting nanocarriers ... 15
2.4.3. Smart stimuli-sensitive carriers ... 16

2.5. Viral and synthetic oligonucleotides as adjuvants ... *17*
2.5.1. Virus-derived Oligonucleotides ... 17
2.5.2. Synthetic oligonucleotides ... 18

2.6. Recent developments in DC vaccines ... *19*

2.7. Aim of the study ... *20*
2.7.1. Composition of the nano vaccine ... 20

3. Material ... 24

3.1. Laboratory Devices ... *24*

3.2. Buffers and Solutions ... *24*

3.4. Expendable Materials ... *25*

3.5. Antibodies ... *25*

3.6. Reagents and chemicals ... *26*

3.7. Oligonucleotides ... *26*

3.8. Cell Culture ... *27*

3.9. Mice ... *27*

3.10. TLR-2 -/- Mice *28*

4. Methods **29**

4.1. Obtaining murine bone marrow-derived dendritic cells (BMDC) *29*

4.2. BMDC activation assays with liposomes and oligonucleotides *29*
4.2.1. Treatment of BMDC (WT) with TLR2-targeting liposomes CL419/CL553 29
4.2.2. Treatment of BMDC with synthetic ODN in a soluble form or in combination with the transfection agent jetPEI 30
4.2.3. Treatment of BMDC (WT) with synthetic ODN in a soluble form or in combination with the transfection agent DOTAP 30
4.2.4. Treatment of BMDC (WT) with viral oligonucleotides in a soluble form or in combination with the transfection agent DOTAP 30
4.2.5. Treatment of BMDC (WT and TLR2-deficient mice) with nucleic acids complexed with CL419/CL553 31
4.2.6. Staining of the treated BMDC with fluorescent labeled antibodies against surface markers of activated DC 32

4.3. Flow Cytometry *33*
4.3.1. Fluorescent labeled Antibodies used in FACS analysis 33
4.3.2. FACS analysis of the fluorescent labeled BMDC 33

5. Results **33**

5.1. Gating strategy *33*

5.2. Activation of BMCD by TLR2-targeting liposomes *34*

5.3. Activation of BMDC by ODNs in a soluble form and complexed with jetPEI or DOTAP *37*

5.4. Activation of BMDC by virus-derived ODN *40*

5.5. Activation of BMDC with ODN and virus-derived oligonucleotides complexed with CL419/CL552 .... *42*
5.5.1. Activation of BMDC with ODN's complexed with CL419/CL552 42
5.5.2. Activation of BMDC with viral oligonucleotides complexed with CL419/CL552 44

5.5. Activation of BMDC with the best performing nucleic acids complexed with CL419/CL552 .... *44*
5.6.1. Trials including BMDC obtained from WT and TLR2-deficient mice 47

6. Discussion **50**

6.1. Summary of the results *50*

6.2. Implications of the results *52*

6.4. Limitations of the interpretation *53*

6.3. Reflection of the results in the context of the current scientific knowledge *53*

6.5. Outlook *55*

Citations **58**

Appendix **63**

1. SDS Page **63**

2. Additional figures from the results **63**

2.1. ODN's complexed with jetPEI *63*

2.2. Viral oligonucleotides in complexes with CL419/CL553 and DOTAP *65*

Curriculum Vitae **68**

List of Abbreviations

AP-1	activator protein 1
APC	antigen presenting cell
Ag	antigen
BMDC	bone marrow derived dendritic cells
bp	base pair
CD	cluster of differentiation
CDP	common DC precursor
CDS	cytosolic DNA sensor
CLR	C-type lectin receptor
CTL	cytotoxic T-lymphocyte (T-cell)
DC	dendritic cells
cDC	conventional/classical dendritic cell
pDC	plasmacytoid dendritic cell
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescent activated cell sorting
FcR	Fc receptor
FSC	forward scatter
GM-CSF	granulocyte macrophage colony stimulating factor
IKK	IκB kinase
IL	interleukin
ILC	innate lymphoid cells
IRAK	interleukin-1receptor-associated kinase
IRF3	interferon regulatory factor 3
i.v.	intravenous
KO	knockout
LPS	lipopolysaccharide
µg	microgram
µl	microliter
MAPK	mitogen-activated protein kinase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex

MyD88	myeloid differentiation primary response protein 88
min	minute
ml	milliliter
MPS	mononuclear phagocyte system
NF-κB	nuclear factor-kappa B
NK	natural killer cells
nm	nanometer
ODN	oligodeoxynucleotide
OVA	ovalbumin
pm	picometer
PBS	phosphate buffered saline
PE	phycoerythrin
PEI	polyethylenimine
PFA	paraformaldehyde
PLGA	poly(d,l-lactic-co-glycolic acid)
RT	room temperature
SD	standard deviation
SSC	sideward scatter
STING	stimulator of interferon genes
TBK1	TANK-binding kinase 1
TAK1	transforming growth factor-β-activated kinase 1
TC	T cell (T-lymphocyte)
TCR	T cell receptor
Th	T helper cell
Treg	regulatory T cell
TME	tumor microenvironment
TNF	tumor necrosis factor
TLR	toll like receptor
TRAF6	TNF receptor-activated factor 6
WT	wildtype

List of figures

Fig. 1: Hematopoiesis: Development of hematopoietic stem cell to different mature cell types 1

Fig. 2: Antibody isotypes: Schematic representation of the five different antibody isotypes present in mammals 6

Fig. 3: Two signal model of T-cell activation: Signal 1 is contributed by antigen-presenting MHCII via TCR. Signal 2 is mediated by costimulatory molecules that either function in a co-stimulatory or co-inhibitory way and therefore yield a balanced T-cell response. 9

Fig.4: STING-dependent pathway: Intracellular dsDNA is sensed by different CDS that activate STING, which yields a TBK1/IRF3 dependent production of Type 1 IFN. 18

Fig.5: TLR signaling: After TLR activation, MyD88 activates interleukin (IL)-1 receptor-associated kinase (IRAK), which associates with TRAF6. TRAF6 activates TAK1. Activated TAK1 then activates IKK complex which activates the NF-κB pathway. Also, TAK1 activates the MAP kinase pathway. NF-κB and AP-1 induce the expression of proinflammatory cytokines. 19

Fig. 6: Molecular structure of CL419 22

Fig. 7: Molecular structure of CL553 (PamadiFectin^{TM}) 22

Fig. 8: Gating strategy for BMDC in FACS analysis: In the first step all viable cells were gated plotting SSC-A versus FCS-A. In the second step all cell duplets are sorted out by plotting FSC-H versus FCS-A. In the third step, only CD11c positive cells (e.g. DC) that showed a high expression of MHCII are selected. 33

Fig. 9: Example of primary FACS data: measurement of fluorescence intensities of different surface markers expressed on BMDC that were either untreated (resulting in a low expression of surface markers) or treated with LPS (resulting in a high expression of surface markers) compared to BDMC treated with CL419 or CL553. 34

Fig. 10: BMCD treated with CL419/CL553: Dose dependent upregulation of MHCII, CD80, CD86. 37

Fig. 11: BMCD treated with ODN D-SL01 (dark green), ODN D-SL03 (orange) and ODN 1826 (dark blue) in a soluble form and complexed with DOTAP (dotted bars): upregulation of MHCII, CD80, CD86. 40

Fig. 12: BMCD treated with ISD (dark yellow), VACV-70 (violet), HSV-60 (red) in a soluble form and complexed with DOTAP (dotted bar): upregulation of MHCII, CD80, CD86. 42

Fig. 13: BMCD treated with ODNs (ODN D-SL01 (dark green), ODN D-SL03 (orange), ODN 1826 (dark blue) in a soluble form and complexed with DOTAP (dotted bars) or CL419/CL553 (striped bars): upregulation of MHCII, CD80, CD86. 44

Fig. 14: BMCD treated with the best performing nucleic acids (ODN 1826 (dark blue), HSV-60 (red)) complexed with CL419 (vertically striped bars)/CL553 (horizontally striped bars): upregulation of MHCII, CD80, CD86...47

Fig. 15: Comparison of WT (light blue) and TLR2 deficient (light green) BMCD treated with the best performing nucleic acids (ODN 1826, HSV-60) complexed with CL419/CL553: upregulation of MHCII, CD80, CD 86...49

Fig. Appendix 1: SDS Page of Complexed formed by CL419 and pDNA plus controls (only pDNA, only CL419, markers and internal control (JetPEI)) performed by Matthias Bros. 63

Fig. Appendix 2 (see next page): BMCD treated with ODN D-SL01 (dark green), ODN D-SL03 (orange), ODN BW006 (yellow), ODN 2395 (dark red), ODN M32 (purple) in a soluble form and complexed with jetPEI (dotted bars): upregulation of MHCII, CD80, CD86. 63

Fig. Appendix 3: BMCD treated with viral oligonucleotides (ISD (dark yellow), HSV-60 (red), VACV-70 (violet)) in a soluble form and complexed with DOTAP (dotted bars) or CL419/CL553 (striped bars): upregulation of MHCII, CD80, CD86. 66

1. General Introduction – Immune system

The immune system is the body's defense system against infectious microorganisms, i.e. bacteria, viruses, fungi and parasites. There is an innate and an adaptive immune system which are no independent systems. They interact and work simultaneously and synergistically.

The innate immune system is the naturally present in the body without prior exposure to pathogens. Its response is not antigen-specific and therefore quick. Owed to that, the immune response of the innate immune system is not as potent as the adaptive immune response. The adaptive immunity is the acquired, specific immune response to a distinct pathogen and therefore only present after exposure. Due to that, he adaptive immune response is slower but more potent and able to create a immunological memory [1]. Moreover, the immune system can be subdivided into a humeral and a cellular system, the humeral system containing complement (innate immune response) and antibodies (adaptive immune response) and the cellular system containing different blood cells deriving from the bone marrow (fig. 1).

The immune system is not only able to fight infections but has different functions in the human body. It can also detect certain tumor cells which can either result in inducing immune tolerance or activation of the immune system [2]. Disfunctions of the immune system can lead to different pathological phenomena. The immune response to endogenous and external harmless antigens can be the cause of allergies [3]. An excessive immune response may predispose for the development of autoimmune diseases [4].

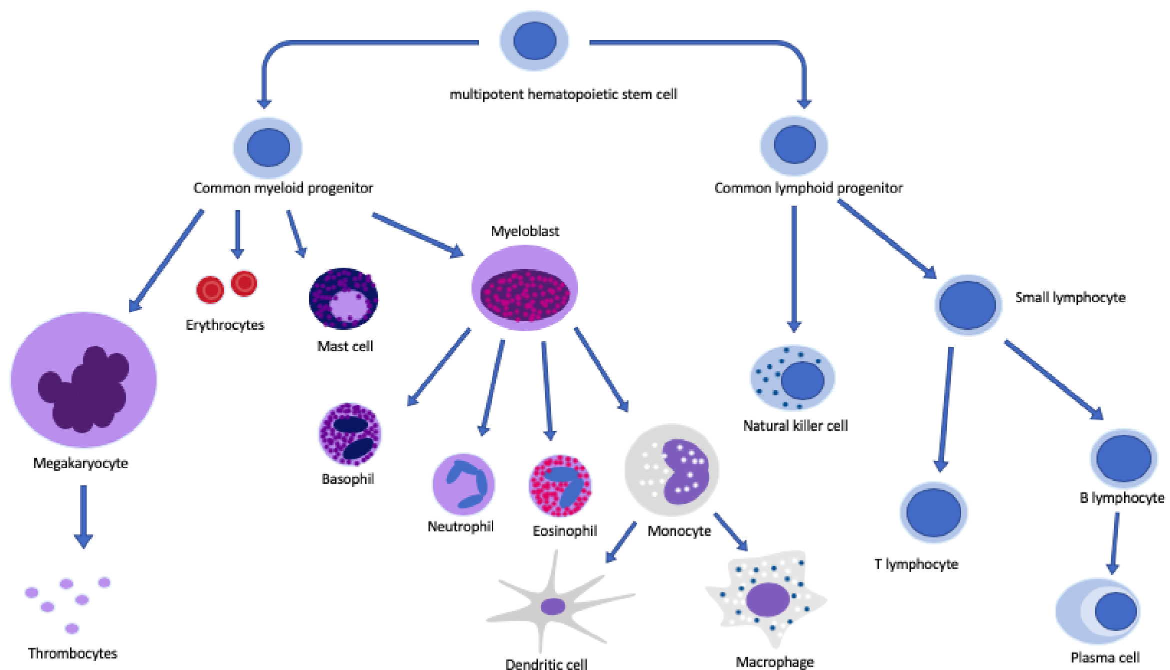


Fig. 1: Hematopoiesis: Development of hematopoietic stem cell to different mature cell types

1.1. Innate immune system

There are different mechanisms the innate immune system uses to protect the body. For one there are physical barriers such as skin and mucous membranes (e.g. intestines, lungs) as well as physiological barriers like temperature and pH value (e.g. in the stomach). Furthermore, there are several chemical barriers which include enzymes and complement. Various types of immune cells are involved in the innate immune response. Cells that take part in the direct immune response are macrophages, dendritic cells (DC), mast cells, granulocytes (basophils, eosinophils, and neutrophils), natural killer (NK) cells and innate lymphoid cells (ILC) [5].

Macrophages, neutrophils and DC are important phagocytes of the innate immune response. Phagocytes are able to engulf and digest pathogens in order to induce their elimination and to present derived peptide antigens to T cells of the adaptive immune system [6]. The cells of the innate immune system recognize unspecific pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) via pattern-recognition receptors (PRRs) [7]. An example for PAMPS is LPS, which is a component of bacterial cell walls and is often used for stimulation of immune cells. The detection of PAMP's initiates cell activation, followed by the production of different chemical mediators like TNFα, IL-1 and IL-6 [5]) is initiated.
Cytokines initialize the adaptive immune response by cell-to-cell communication. Therefore, they are an important link between the innate and the adaptive immune system. Also, they induce local cell responses to inflammatory tissue. A dysregulated production of cytokines is associated with autoimmune diseases, and therefore these constitute targets for therapeutic approaches [5].

1.2. Adaptive immune system

The adaptive immune response orchestrates the acquired, antigen-dependent response to pathogens. Thus, it takes more time to act than the innate immune response. It includes the production of antibodies and may create an immunologic memory [8]. Consequently, a subsequent exposure to the same antigen yields a quicker and more efficient immune response. The adaptive immune system not only detects antigens but can distinguish between "self" and "non-self".

T-cells are part of the adaptive immune system and are activated by APC (antigen presenting cells), e.g. DC. After contact with native antigen, B cells differentiate into plasma cells and produce antigen-specific antibodies [5].

1.2.1. APC

APC are a heterogeneous group of cells, including DC,
macrophages and B cells. They are an important link between the innate and the adaptive immune system as they are able to process antigens and present them to of T-cells. APC express major histocompatibility complexes (MHC) on their surface. Those present antigen fragments to T cells which can be recognized by the corresponding TCR. APC are able to present antigen fragments of intracellular pathogens such as viruses, as well as phagocytosed antigen fragment of extracellular pathogens, such as bacteria.
There are class I MHC and class II MHC. MHCI is found on all nucleated cells and presents endogenous peptides, whereas MCHII is mainly found on professional APC such as DC and macrophages, and presents peptides of exogenous origin [5].

1.2.2. T-cells and their subsets

T-cells derive from hematopoietic stem cells in the bone marrow and then migrate into the thymus for maturation. Each thymocyte expresses a T cell receptor (TCR) on their surface that binds a particular antigen at high affinity. T cells in the periphery therefore recognize processed antigens presented via MHC on APC. Activation of T cells is induced in the presence of co-stimulatory receptors (e.g. CD80, CD86 on APC). When activated, T cells will rapidly proliferate and differentiate into effector cells exerting different functions. $CD8^+$ cytotoxic T-cells are involved in the direct elimination of pathogens, $CD4^+$ Ts helper cells (Th) support the activation of B- and T-cells, whereas regulatory T-cells (Treg) downregulate the immune response, limiting tissue damage [9].

1.2.2.1. T helper cells (Th)

$CD4^+$ Th cells produce cytokines in order to enhance the B- and T-cell response to a pathogen. They recognize antigens presented on MHCII molecules. After activation Th cells differentiate into distinct subclasses (e.g. Th1, Th2, Th9, Th17, Th22) that are characterized by different

cytokine profiles and exert different functions. Their differentiation is dependent on various factors, including cytokines, costimulatory molecules, receptor-mediated signal transduction pathways and transcription factors [10]. Th1 develop in an IL-12 rich environment and produce mainly IL-2 and IFN-γ, whereas Th2 under the influence of IL-4produce IL-4, IL-5, IL-10 and IL-13. Cytokines of Th1 cells yield cell dominated immune responses. In contrast, Th2 cells produce cytokines that enhance antibody production [11]. Th1 as well as Th2 cells can yield immunopathology. An excessive Th1 response will produce tissue damage whereas Th2 rather provokes allergic reactions. Th17 are the main source of cytokines of the IL-17 family and are considered to play a key role in the induction and mediation inflammation and autoimmunity. Th22 cells have been found to play a role in the pathogenesis of autoimmune skin diseases like SLE and psoriasis and neuroinflammatory diseases e.g. MS. The effects of TH22 cells are mainly mediated by IL-22, a member of the IL-10 family. TH9 cells are characterized by their IL-9 production and seem to be involved in the pathogenesis of asthma. This can be attributed to the important role of IL-9 in mucus production and activation of mast cells and eosinophiles [12].

1.2.2.2. Regulatory T-cells

Regulatory T-cells (Treg) are a subset of $CD4^{+}CD25^{+}$ T-cells. Treg are involved in maintaining self-tolerance but also in the prevention of autoimmune diseases [10] and allergies [13]. In contrast to Th cells, Treg suppress immunological reactions and therefore contribute to immune tolerance. Therefore, Treg are an important counterpart to Th cells for immune homeostasis. In mouse, the transcription factor Foxp3 is essential for Treg differentiation [14]. Several Treg populations produce high quantities of IL-10 which might not directly function in an immunosuppressive way, but rather through its influence on APC downregulation the expression of MHC molecules and other co-stimulatory molecules (CD80/CD86). It could be involved in the maintenance and expansion of Treg [12].

1.2.2.3. Cytotoxic T-cells (CTL)

CTL play an important role in the elimination of infected or malignant cells (e.g. tumor cells). They recognize antigens presented on MHCI molecules and release cytotoxic effector proteins upon contact with a target cell. These cytotoxic proteins include perforin, granzymes and granulysin which induce apoptosis. CTL also produce cytokines including TNF- α and IFN-γ which induce MHCI expression and restrain viral replication [15].

Clinically, CTL are an important target for research. Tumor cells express certain antigens on MHC molecules that can be detected by CTL. Somehow some tumor cells manage to evade the recognition by CTL. The research regarding CTL therefore is aimed at (re)activating tumor-specific CTL, in order to enhance the elimination of cancer cells [16].

1.2.3. B-cells

B cells derive from hematopoietic stem cells in the bone marrow. They express distinct antigen-specific receptors on their cell surface (B cell receptor, BCR) and then migrate into lymphatic tissues/organs. B cells recognize native protein antigens via the BCR. When activated, B cells differentiate into plasma cells and produce antigen-specific antibodies [5]. Hence, B cells are part of the humoral immune response. Some B cells develop into memory cells that yield an early response in case of reinfection [9]. Different isotypes of antibodies exist (IgM, IgD, IgG, IgE and IgA) that execute different functions (fig. 2) [17]. IgM antibodies are expressed on the surface of immature B-cells. Upon antigen contact, IgM pentamers are secreted by B-cells and provide an early response for a wide antigen range. IgD is present on the surface of B-cells and the expression correlates with the activation the cell. IgD is not a secretory immunoglobulin. IgG is the most common immunoglobulin (80-85%) found in the peripheral blood. It is produced by plasma cells and mediates opsonization and neutralization of antigens as well as complement activation. IgA is secreted into the mucus and provides mucosal immunity. Defense against parasites is provided by IgE. IgE also contributes to the pathogenesis of allergies by engaging mast cells and eosinophils [17].

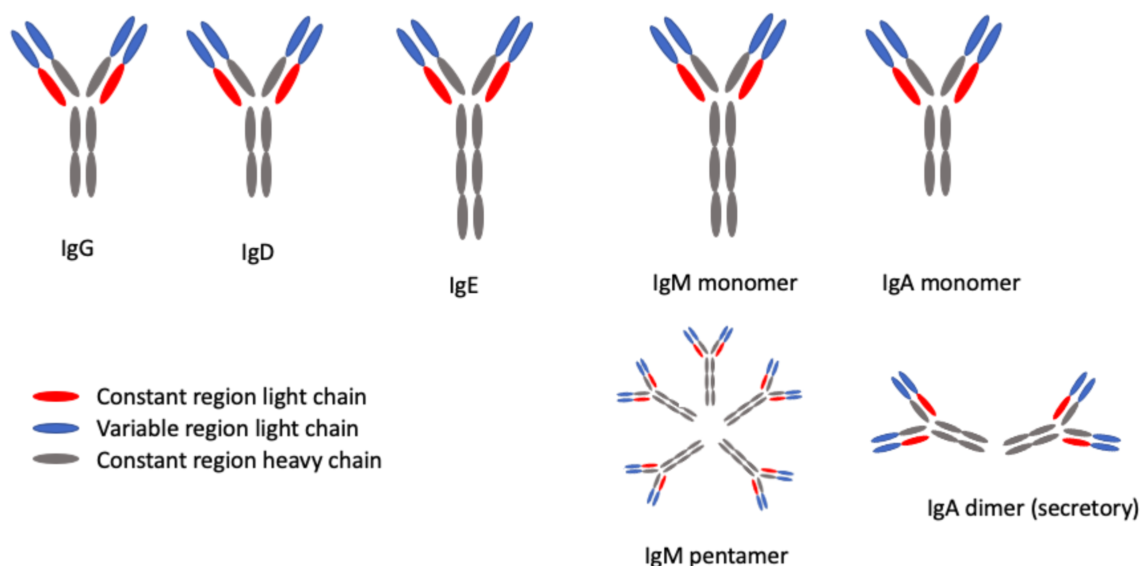


Fig. 2: Antibody isotypes: Schematic representation of the five different antibody isotypes present in mammals

1.3. Dendritic Cells

DC originate from hematopoietic stem cells in the bone marrow. They are crucial for the initiation of an adaptive immune response. In the periphery immature DC recognize, internalize and process pathogens and undergo maturation. Mature DC then migrate to secondary lymphatic organs where they present processed pathogen-derived antigen to T cells via MHC molecules and therefore function as APC [18]. They play an important role in the induction of primary immune responses, as well as the induction of immunological tolerance [19].

1.3.1. Ontogeny of DC

DC are a member of the mononuclear phagocyte system (MPS) that also includes macrophages and monocytes. They all have functional and phenotypical characteristics in common.
Guilliams et al. suggested 2014 that DC may be distinguished by their ontogeny and by their location, function and phenotype [20]. Regarding ontogeny, conventional or classical DC (cDC) and plasmacytoid DC (pDC) derive from a common DC precursor (CDP). Furthermore, there are two lineages of cDC, namely type 1 cDC (cDC1s) which are CD8α^+ and CD103$^+$, and cDC2s that express CD11b and CD172a. cDC derive from precursor cDC (pre-cDC) that develop from CDPs in the bone marrow. Pre-cDC migrate into periphery and mature into cDC. pDC mature in the bone marrow via a pre-pDC intermediate stage. cDC are claimed to function as APC and therefore as regulators of Th cell immune responses. pDC are also referred to as interferon producing cells (IPC) and have a morphological similarity to plasma cells.
The maturation of all DC populations is dependent on the cytokine FMS-like tyrosine kinase 3 ligand (FLT3L) [21]. The subsequent development of the different DC subsets is dependent on distinct transcription factors. cDC1 development is regulated by IFN-regulatory factor 8 (IRF8), DNA-binding protein inhibitor ID2, basic leucine zipper transcriptional factor ATF-like 3 (BATF3) and nuclear factor interleukin (IL)-3-regulated protein (NFIL3), whereas cDC2 development is provoked by RELB, PU.1, recombining binding protein suppressor of hairless (RBPJ) and IRF4 [20]. pDC development depends on the expression of transcription factor E2-2 (also known as TCF4) [22].

Monocytes have a certain plasticity that enables them to develop DC characteristics under circumstances of inflammation. Nevertheless, they are no DC precursors in a steady state environment [23].

1.3.2. Surface Markers

Flow cytometry has allowed to distinguish cells by means of cell lineage-characteristic surface markers. Murine DC can be identified by their CD11c and MHCII expression [20]. MHCII is expressed by DC at large quantities after activation. Depending on their location DC express different surface markers (e.g. $CD207^{+}$ cDC1 in the dermis).
Phagocytosis of pathogen particles induces the expression of different costimulatory molecules on the surface of DC (e.g. CD80 and CD86) that are essential for T-cell activation [23].

1.3.3. Toll-like receptors (TLR)

The innate immune system relies on the recognition of pathogen-associated molecular patterns (PAMPs). Therefore, cells of the innate immune system, such as DC, express a limited number of germline-encoded receptors, so-called pathogen recognition receptors (PRRs). The TLR family is a well-known example of PRRs that are able to detect a number of different PAMPs. Different DC subsets express distinct classes of TLR molecules and other PRR on their surface in order to pursue different tasks.
TRLs are related to IL-1 receptors as they possess an intracellular domain composed of toll/interleukin-1 receptor (TIR), however they are characterized by an extracellular portion that contains leucin-rich repeat domains [24]. Until today, up to ten different functional TLRs have been defined that are expressed on different human cells in different quantities and respond to different stimuli. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface, where extracellular pathogens can be detects. TLR3, TLR7 and TLR9 remain in intracellular compartments (e.g. endosomes). They recognize particles presented to them in phagosomes or endosomes [10]. TLR2 and TLR4 for example recognize components of bacterial cell membranes as e.g. LPS, whereas TLR9 is stimulated by unmethylated CpG DNA which is found in bacteria and viruses [25]. Mammalian CpG motifs mostly present themselves methylated. Hence, in contrast to bacterial/viral DNA, it will not be recognized by TLR9.

1.3.3.1. TLR in dendritic cells

Immature DC in the periphery express a number of receptors for antigen capture and uptake, including TLR. DC express different TLR classes, dependent on the subset.
Human pDC express TLR7 and TLR9 and therefore recognize bacterial or viral nucleic acids in the context of an infection or endocytosis, whereas cDC express TLR2 and TLR4 and recognize extracellular pathogens (e.g. bacteria). Accordingly, they produce different classes of cytokines. TLR4, TLR7 and TLR9 lead to the production of similar cytokines, inducing a Th1 response, whereas TLR2 rather leads to a production of Th2 stimulating cytokines [10].
Also, DC migration from the periphery into the lymphatic tissue is induced by TLR activation via down-regulation of tissue homing chemokine receptors (CCR2 and CCR5) and up-regulation of the lymph node-homing chemokine receptor CCR7 [26]. In summery TLR signaling yields the transformation of phagocytic DC into antigen presenting DC.

1.3.4. Interaction between DC and T-cells

After stimulation, DC migrate from the periphery to the T-cell zone of secondary lymphatic organs, where they express high quantities of MHC molecules that present pathogen-derived antigens to T cells via the TCR (signal 1). After uptake of the respective antigen, $CD4^+$ T helper cells respond to peptides presented on MHCII molecules, whereas $CD8^+$ cytotoxic T cells depend on antigen presentation via MHCI, also referred to as cross-presentation.
T-cell activation requires a second signal which is delivered by costimulatory molecules on the surface of DC that interact with distinct receptors (CD28) on T-cells (fig. 3)
Depending on the ratio of co-stimulatory and co-inhibitory receptors, the second signal can either promote or suppress the T-cell response and accordingly yield immune tolerance or immune activation.
The B7/CD28 signaling axis is crucial for T-cell stimulation. B7 (B7-1 or CD80 and B7-2 or CD86) protein are expressed by activated DC. CD28 is expressed on T-cells. Upon positive co-stimulation, IL-2 production and IL-2 receptor expression of T-cells is enhanced, and T-cells differentiate into effector cells.
Negative stimulation can be induced by different molecules, such as cytotoxic T lymphocyteantigen-4 (CTLA-4) interacting with CD80/CD86. It limits the initial T-cell response and induces tolerance. The coexistence of activating and downregulating stimuli on immune cells is essential for immunological homeostasis [10].

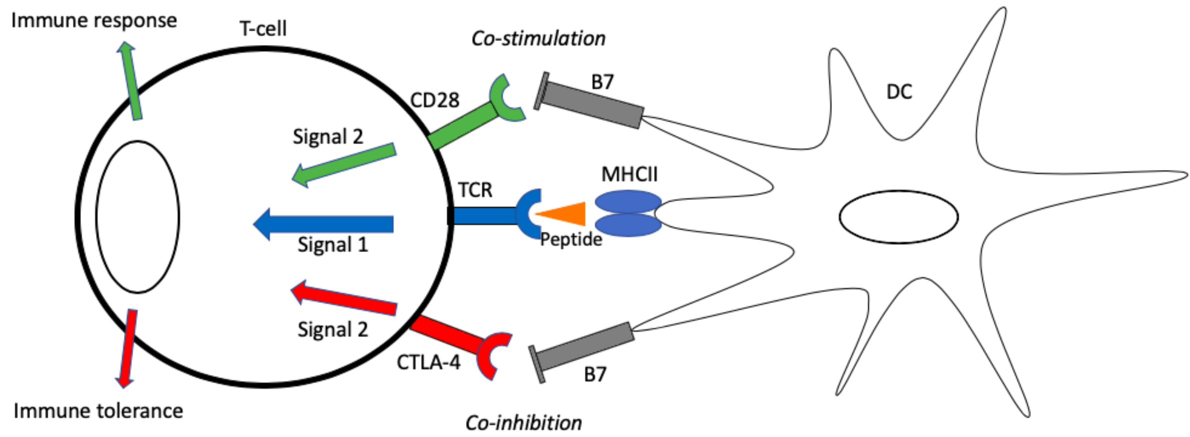


Fig. 3: Two signal model of T-cell activation: Signal 1 is contributed by antigen-presenting MHCII via TCR. Signal 2 is mediated by costimulatory molecules that either function in a co-stimulatory or co-inhibitory way and therefore yield a balanced T-cell response.

2. Discussion of Literature - Cancer Immunotherapy

Cancer immunotherapy is becoming a more popular approach which designates the strategy to attack tumor cells by stimulating immune cells rather than using chemotherapy that attacks not only tumor cells but all frequently dividing cells. Immunotherapy itself can be divided into an unspecific class, e.g. check point inhibitors or modified cytokines, and an antigen-specific class [27]. The latter is aimed to provoke a tumor-specific immune response by programming immune cells to recognize tumor-specific antigens and to kill the respective cells [28]. In this regard, T cells can be targeted directly by tumor-specific vaccines (CAR-T cell therapy) or be stimulated via MHCI/MHCII interaction with APC, particularly DC, that were priorly activated through vaccination. In contrast to the classical vaccines designed to prevent infections, tumor vaccines are hereby used in a therapeutic manner [29].

2.1. DC as a target for immunotherapy

DC exert an important function as immune regulators and are therefore involved in the induction and regulation of anti-tumor immune responses [30]. DC possess the capacity to uptake particles through different kind of mechanisms, depending of the particles size, charge, surface properties and receptor ligands [31]. Immature DC in the peripheral tissue like the skin internalize and process antigen and when activated migrate through the lymphatic vessels to the draining lymph nodes where matured DC present antigen to T cells via MCH molecules. In tumor patients endogenous DC show certain dysfunctions, induced by immunosuppressive factors present in the tumor microenvironment, which can reduce their activity and therefore prevent the patient's own immune response against the tumor [32]. Consequently, they might be suitable for targeted anti-tumor immunotherapy.

Anti-tumor vaccines targeting DC are designed to provoke a tumor specific T cell response. With the very selective targeting of specific antigen containing tumor cells, the toxicity is lower compared to conventional therapeutic approaches. Another advantage of anti-tumor vaccination would be, that after antigen contact, distinct T cells will develop into memory cells, inducing a remaining surveillance against those tumor cells [28].

Considering the ability of DC to not only initiate and enhance immune responses, but their ability of inducing immune tolerance, DC also appear to be suitable for approaches regarding the prevention or immunotherapy of autoimmune diseases [33]. In autoimmune diseases, the loss of self-tolerance plays a crucial part in the pathogenesis. Initially, DC in the thymus present self-antigen to T cells, followed by a strict negative selection of autoreactive T cells and

generation of regulatory T cells (Treg), which yields central self-tolerance. There are also several mechanisms through which DC promote peripheral tolerance. In patients with autoimmune diseases defective Treg function is found. In some autoimmune diseases like rheumatoid arthritis (RA) there are distinct autoantigens found, offering a new therapy approach on the basis of autoantigen-specific Treg, inducing a targeted, autoantigen-specific immune regulation [34].

2.2. Limitations of conventional vaccines

Conventional vaccines utilize attenuated or inactivated pathogens, or fractions of pathogens that do not cause the disease, in order to train the adaptive immune system to recognize the very pathogen in case of a subsequent infection [35]. Antigen containing vaccines that included no adjuvants proved to induce mucosal tolerance, which could be helpful in fighting autoimmune diseases, but not fighting infection or cancer [36]. In order to increase the immune response while reducing the quantity of antigen needed, adjuvants containing e.g. aluminum were used since the 1930s [37]. Generally, there are adjuvants predominantly improving the delivery system of associated antigen to APCs (e.g. emulsions or liposomes) and others that rather exert immunostimulatory functions and were deriving from pathogens (e.g. LPS, CpG oligonucleotides, DNA) [38].

These vaccines have been successfully inducing immunity against viral and bacterial infections and would typically be injected intramuscular, subcutaneously or taken orally. Some disease, like the smallpox, were even eradicated through vaccination. Without doubt, conventional vaccines were an enormous gain for humanity in their fight against infectious diseases. Nevertheless, there are certain disadvantages in conventional vaccines like their weak immunogenicity, and therefore the need for multiple administrations, as well as the intrinsic instability in vivo, the toxicity and adverse events like allergic reactions to the protein. Some autoimmune reactions, presenting similar symptoms, namely siliconosis, the Gulf war syndrome (GWS), the macrophagic myofasciitis syndrome (MMF) and post-vaccination phenomena, were associated with certain adjuvants (e.g. infectious agents, silicone, aluminium salts). Shoenfeld and Agmon-Levin suggested to term those conditions sharing a common denominator, “ASIA-Autoimmune/inflammatory Syndrome Induced by Adjuvants” [39]. Nevertheless, it is not implied, that adjuvants are the single cause for autoimmune processes, but rather serve as a trigger in humans that hold a genetic background for developing

autoimmune diseases [40]. Still, there is a need for developing safer adjuvants for the use of vaccines in humans [41].

Today, the demands concerning vaccinations have increased considering challenges like highly variable pathogens, new emerging infectious diseases or therapeutic applications for chronic infections and cancer or a combination of both [42].

A more recent development makes use of DNA vaccines in form of plasmid DNA encoding a specific antigen of the respective pathogen [43]. The plasmid DNA is injected intramuscularly where the muscle cells will encode it and express the antigen which yields an immune response in which local DC may play an important role. Those DNA vaccines offer many advantages over conventional vaccines: they are able to induce a cellular as well as a humoral immune response, they present an excellent safety profile, a high degree of stability and are easier to manufacture [44]. However, DNA vaccines induced only a low immunogenicity in former studies, which has been an obstacle for their use in fighting infections or cancer in humans. Approaches in order to improve immunogenicity have been made by e.g. using different application routes or adding molecular adjuvants [45].

2.3. Advantages of nano-vaccines

Nano-medicine denominates the use of particles at a nano range (10^{-9}) for diagnostic or therapeutic purposes. Nano-particles are designed to interact with cells on a molecular and very specified level [46]. Naturally occurring nano particles are for example viruses (30-50nm). Synthetically produced nano-particles can be made from different materials such as metals, inorganic materials, polymeric materials or lipids [47]. It has been shown, that particles smaller than 10 µm are easily engulfed by phagocytic cells, such as macrophages and DC, which makes them interesting for therapies targeting the immune system [48].
The use of nanotechnologies in the field of vaccination comprises nano-carriers with different properties that execute different functions [49]. Various nano-carriers have been designed to improve and direct the delivery of antigen and/or adjuvant, in order to increase the immunogenicity of the vaccine [48]. Nano-carriers hereby should prevent the antigen to be degraded rapidly, and only induce a short-lived, localized immune response. Moreover, the nano-carrier serves the purpose of presenting the antigen to the immune system, mimicking the function of a pathogen. Some nanocarriers may also be able to control the release of the antigen, to prolong its exposure to the immune system [50].

When only the antigen is incorporated into the nanocarrier the vaccine may fail to have an effect on the stimulation of the immune cells. On this account nanocarriers that co-deliver both antigen and adjuvant have been designed and tested, suggesting that the inclusion of the adjuvant enhances the antigen specific T cell response [51].
Ligands for surface receptors, such as mannose receptor, scavenger receptors, and toll-like receptors (TLR) that are present on APC (e.g. DC) were conjugated to nanocarriers in order to target those receptors specifically for optimized antigen delivery. When stimulated via one of these distinct receptors, upon codelivery of an adjuvant DC engulf the antigen into endosomes for loading onto MHC molecules and stimulation of T-cell responses [52].
Another advantage some nanocarriers offer is the possibility of mucosal vaccine delivery, which has been a challenge due to the natural barriers on mucosal surfaces. Nano-vaccines facilitate the entry into the gut-associated lymphoid tissue and mucosa-associated lymphoid tissues by stabilizing the antigen containing particle throughout the gastrointestinal passage and increasing the uptake [53]. Since many childhood infections occur via the mucosal surface, the mucosal route seems to be of advantage, because it would be needle-free and, additionally to a systemic immunity, induce mucosal immunity. [54].

2.4. Use of different nanocarriers

Nanocarriers can be categorized by their ability to target and stimulate APC. Mainly, there are three different classes: (1) Passively targeting carriers, (2) actively targeting carriers, (3) smart stimuli-sensitive carriers [55]. Recent approaches regarding nanocarrier vaccine systems have been including liposomes, emulsions, cationic nanocarriers and polymer-based nano-delivery systems [48].

2.4.1. Passively targeting nanocarriers

Passive targeting of DC implies the uptake of the nanoparticle through the natural capacity of DC, being able to uptake molecules with certain features via endocytosis. It might not yet be known which distinct receptors play a part in endocytoses of those molecules. Depending on the chemical features of the respective particles, different endocytosis pathways are reportedly used by DC for particle uptake (e.g. phagocytosis). It has been suggested that hereby nanoparticles with a lower diameter (300 nm) have a higher uptake by DC than particles with a higher diameter (1 µm) [56].

2.4.1.1. Liposomes

Liposomes are composed of phospholipid bilayers. Liposomes have been known to target and stimulate immune cells owed to their biochemical properties (e.g. size, potential, composition), acting as immunological adjuvants [48]. Another approach is to conjugate liposomes with a receptor specific antibody or another distinct molecule that severs as a ligand, in order to address a specific receptor and thereby optimize the immune response to the respective antigen [57]. The conjugation with liposomes is enabled by their hydrophilic as well as lipophilic features which allows them to form complexes with both hydrophilic and lipophilic molecules. Hence, molecules with different chemical features such as antigens and adjuvants can be loaded in the same liposome.

Liposomes have been shown to incorporate model tumor antigens [55].

Jerome at al. [58] designed a nano-vaccine incorporating melanoma specific antigen (TRP2) and oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) as an adjuvant (TLR9 ligand) into a liposome (AVE3). The vaccination could yield a T cell response. The most potent response was observed when both the peptide and the adjuvant were encapsulated into the liposome, in contrast to the free antigen or adjuvant alone (i.e. not encapsulated).

Also, their absence of toxicity is favorable for the in-vivo use [55].

2.4.1.3. Cationic Nanocarriers

Cationic nanoparticles are generally better engulfed by APC than neutral or anionic molecules. For example, positively charged gold nanocarriers led to a higher uptake efficiency by DC in human [59]. However, cationic particles tend to aggregate at the injection site and therefore are not drained to the lymphatic vessels effectively. Also, cell uptake and stimulation are not cell-type specific.

Cationic liposomes are the best investigated group of cationic nanocarriers. Studies using cationic liposomes containing tumor antigens like OVA peptide or adjuvants such as CPG oligonucleotides yielded better anti-tumor immune response then the soluble antigen itself [55].

2.4.1.4. Polymer-based nano-delivery systems

The big advantage of polymer-based nanocarriers is the prolonged, gradual release of the antigen and adjuvant rather than a targeted delivery system [60]. Some polymer-based particles, namely Poly(d,l-lactic-co-glycolic acid) (PLGA), have been shown to be engulfed by DC after

intradermal injection and to stimulate DC without additional adjuvants [61]. In-vitro PLGA nano-carriers increased the expression of MHCII and CD80/CD86 on APC. PLGA, being a commonly used biodegradable polymer, has been used to encapsulate tumor-associated antigens in former studies [55]. Solbrig et al. published a study showing that PLGA encapsulated OVA and gp100 peptide. When encapsulated, the antigenicity was increased compared to the soluble antigen [62].

2.4.2. Actively targeting nanocarriers

By targeting surface receptors on DC specific binding and uptake of nano-carriers can be obtained. Active targeting of APC, especially DC, is yielded by receptor/ligand interaction in order to induce an antigen presentation via MHC molecules which may yield a cellular immune response. There are several receptors present on DC that may be targeted [55].

2.4.2.1. Mannose receptor targeting nanocarriers

The mannose receptor is a C-type lectin receptor (CLR), all of which are calcium-dependent receptors that share a common carbohydrate recognition domain. Nevertheless, mannose receptors are not only present on DC, but on many types of cells, such as monocytes, macrophages and distinct endothelial cells. Still, complexes formed by mannose and a tumor antigen have been successfully tested in preclinical studies. Furthermore, mannose containing nanocarriers (in this case liposomes) complexing antigen have been included in studies that suggested an enhanced uptake and activation of DC [63].

2.4.2.2. Fc receptor targeting nanocarriers

Fc receptors (FcR) are membrane receptors that bind the constant part of antibodies of different isotypes. Due to their affinity they are divided into subgroups determined by the respective immunoglobulin (Ig) class. IgG-coated liposomes have been used to target those receptors and where successfully taken up by DC and the contained antigens were presented to T cells [64]. Furthermore, Suzuki et al. showed that OVA peptide encapsulated by liposomes that were conjugated with IgG prevented the growth of OVA-expressing lymphoma cells in mice to a higher extent as compared to non-targeted liposomes or soluble OVA [65].

2.4.2.3. CD11c/CD18 and DEC-205 receptor targeted particulate carriers

CD11c/CD18 and DEC-205 are believed to play an important role in the process of antigen capture and presentation. The heterodimer CD11c/CD18 is a member of the leukocyte ß2 integrin receptor family and in mouse is mainly expressed on DC and binds several ligands, including bacterial lipopolysaccharide (LPS), complement or ICAM-2 [66]. DEC-205 is an endocytic type I C-type lectin-like molecule that is expressed at high levels on cortical thymic epithelium and different subsets of DC. DEC-205 can shuttle internalized antigen for presentation on MHC class II and I (cross-presentation). In a study by van Broekhoven et. al liposomes were prepared containing OVA peptide. Antibody fragments specific for either CD11c or DEC-205 were attached to the surface of the liposomes. The results suggest that targeting DC this way induces a significant immunotherapeutic effect. [67].

2.4.3. Smart stimuli-sensitive carriers

Smart particle carriers are aimed at delivering antigen into the cytosol of DC making extracellular particles accessible to intracellular processing and presentation on MHCI molecules yielding a CTL response [55]. This way the importance of the usual MHCII presentation and T helper cell response induced by exogenous particles is reduced. Those smart carriers include pH-sensitive particle carriers, fusogenic systems, or virosomes/virus like particles.

2.3.4.1. pH-sensitive particle carriers

pH-sensitive carriers are designed to release the incorporated antigen in an environment with a low pH value. Conveniently, those carriers are mostly taken up by DC in endosomes that have a lower pH than the cytosol. By disruption of the endosome, which is enabled by certain characteristics of the carrier (e.g. high basicity), the respective antigen is released into the cytosol and can be bound to and presented by MHCI molecules.
Zhou et al. showed that pH-sensitive particle carriers yielded a better CTL response than pH-insensitive particle carries in vitro as well as in vivo. They used OVA encapsulating liposomes that were either pH-sensitive or -insensitive to treat murine DC subsequently used to induce a CTL response in vitro [68].

2.3.4.2. Fusogenic systems

Fusogenic systems can be obtained by attaching virus-accessory proteins to liposomes, conserving the capability of the virus to confer membrane fusion.
Yoshikawa et al. described the utility of fusogenic liposomes as tumor cell-lysate delivery carriers for ex vivo DC vaccination and direct in vivo immunization against murine B16 melanoma. Both strategies showed anti-tumor effects, the ex vivo trial having the more potent effect on tumor prophylaxis [69].
A new concept of combining both strategies, pH-sensitive and fusogenic liposomes, has been tested by Yuba et al.. In that approach liposomes exerted fusion ability at a low pH value. Those liposomes, incorporating OVA peptide, were efficiently taken up by murine DC in vitro and delivered the antigen into the cytosol, followed by MHCI presentation [70].

2.3.4.3. Virosomes

Virosomes are modified viral envelopes containing virus-derived proteins in their membrane which allows them to fuse with their target cells. This way they are able to deliver incorporated antigen into the cytosol of DC or other target cells. In a study by Bungener et al. it was shown that fusion active virosomes delivered incorporated OVA peptide more efficiently into the cytosol of DC, followed by MHCI presentation, then fusion inactive virosomes and FcR targeted liposomes [71].

2.5. Viral and synthetic oligonucleotides as adjuvants

Vaccines consisting only of purified protein antigen have been found to induce no efficient immune response. Hence, effective vaccines require an adjuvant to stimulate APCs. Immune stimulation through oligonucleotides is performed by the innate immune system through PRRs and serves the purpose of pathogen and anti-tumor protection.
Viral oligonucleotides and synthetically produced CpG-rich ODNs both have proven to be potent stimulators of APCs.

2.5.1. Virus-derived Oligonucleotides

There are numerous intracellular sensors for pathogen-derived (oligo)nucleotides (cytosolic DNA sensors (CDSs)) that induce different immune responses. One example is the STING-

dependent pathway, that is induced by dsDNA. dsDNA is recognized by CDSs like cGAS, DDX41, DAI and IFI16 yielding STING-dependent, cellular and humoral immune responses, including T cell as well as B cell responses [72].

2.5.1.1. STING-dependent pathway

After recognition by CDS, cytosolic dsDNA induces STING-dependent production of type 1 IFN. STING stimulates phosphorylation of interferon regulatory factor 3 (IRF3) by TBK1 (TANK-binding kinase 1). Phosphorylated IRF2 triggers the type 1 IFN production (e.g. IFN-β) (Fig. 4) [73].

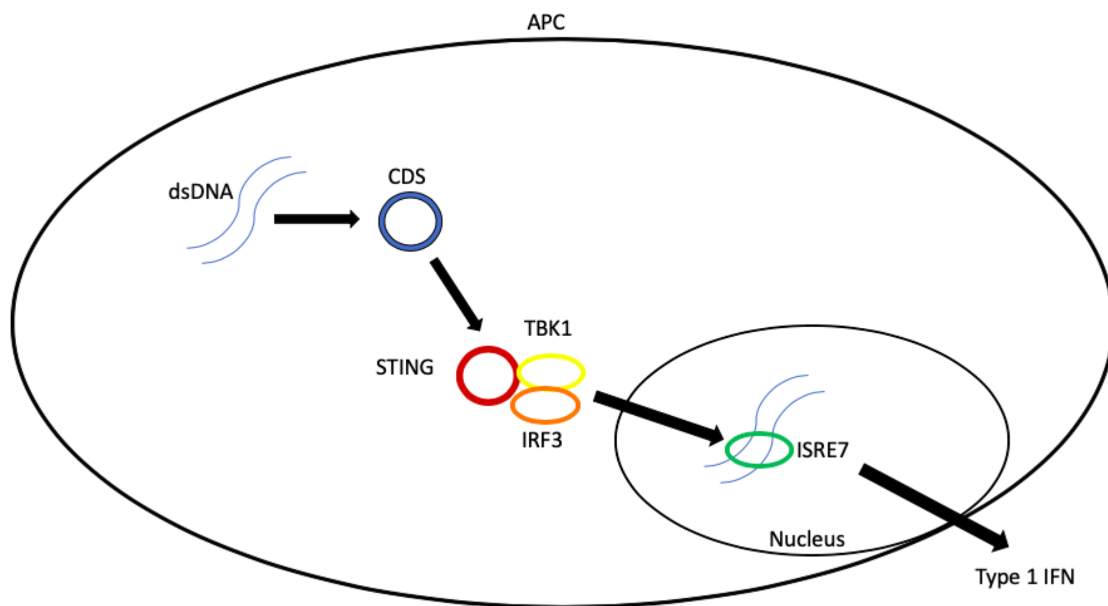


Fig.4: STING-dependent pathway: Intracellular dsDNA is sensed by different CDS that activate STING, which yields a TBK1/IRF3 dependent production of Type 1 IFN.

2.5.2. Synthetic oligonucleotides

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants, inducing an immune response to the co-administered antigen [74]. Non-methylated CpG motifs are present at a high frequency in bacterial DNA, but rare in human DNA. TLR9 receptors recognize those highly preserved motifs at unmethylated state [75].

2.5.2.1. TLR signaling pathways

TLR signaling is highly related to IL-1 signaling, regarding their receptors showing a homology in their intracellular domain (TIR domain). MyD88 (myeloid differentiation primary response protein 88) is a common adapter protein within the IL-1 and TLR signaling pathways[76]. MyD88 contains a death domain, as well as a TIR domain that engages the cytosolic TIR domain of TLR [76] (fig. 5). Upon ligand stimulation, MyD88 recruits a death domain containing interleukin (IL)-1 receptor-associated kinase (IRAK), through the interaction of their death domains. IRAK-1 associates with TRAF6, which activates TAK1. Activated TAK1 then activates IKK complex which lead to a translocation of NF-κB into the nucleus. TAK1 simultaneously activates the MAP kinase pathway, which results inactivation of AP-1. NF-κB and AP-1 induce the expression of proinflammatory cytokines, such as IL-6, IL-12 and TNF-a [77].

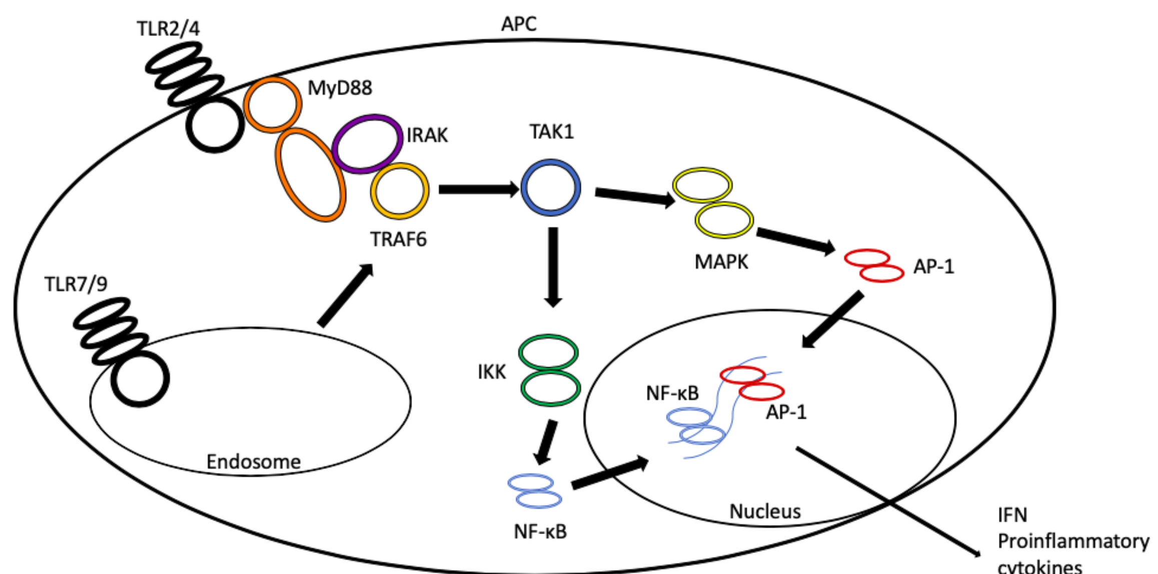


Fig.5: TLR signaling: After TLR activation, MyD88 activates interleukin (IL)-1 receptor-associated kinase (IRAK), which associates with TRAF6. TRAF6 activates TAK1. Activated TAK1 then activates IKK complex which activates the NF-κB pathway. Also, TAK1 activates the MAP kinase pathway. NF-κB and AP-1 induce the expression of proinflammatory cytokines.

2.6. Recent developments in DC vaccines

So far, the safety and immunogenicity of DC vaccines has been proven in clinical trials. Clinical trials have also indicated a potentially high anticancer efficacy. Nevertheless, the clinical responses in regard to personalized vaccines comprising patient-derived DC manipulated ex vivo, have not yet shown a significant success. This could partially be attributed to the

immunosuppressive tumor microenvironment (TME) that limits DC and effector cell function. DC and T cells that infiltrate tumors, migrating into the TME, become tolerogenic [78].
It is suggested by Belderbos et al that the inhibitory effect of the immunosuppressive TME on the efficacy of anti-tumor DC-vaccines could be overcome by combining the DC therapy with other tumor therapies, such as chemotherapy, checkpoint therapy or radiation. Thereby, the additional treatment is aimed to target immunosuppressive cell subsets in the TME, inducing immunogenic cell death or blocking inhibitory molecules [79].
Trials, manipulating DC in vivo seem to be promising [80].
Multiple sites of the body can be targeted where DC are in their natural environment.
Many different strategies for DC targeting anti-tumor vaccines, including ex vivo and in vivo DC targeting and different composition of vaccines, have been suggested. Each approach bears advantages and disadvantages for the heterogenous population of tumor patients. Baldin et al. suggest to optimize the use of DC vaccines by using more individual approach based on each patients tumor biology [81].

2.7. Aim of the study

In general, this work pursues the characterization of commercial cationic liposomal carriers and suitable adjuvants, inducing a DC response. In this context the different liposomes and adjuvants have been tested according to their ability to induce a potent immune response. Furthermore, it was investigated if the substances could yield synergistic effects on the DC activation, when used in a complexed form, acting as an immunogenic DC vaccine.

2.7.1. Composition of the nano vaccine

In general, there are three components required in order to induce a tumor-specific, DC controlled immune response, i) a tumor-associated antigen, ii) a DC activating entity i.e. an adjuvant, and iii) and a targeting moiety. A nano-carrier can encapsulate the antigen and the adjuvant. The targeting component can be attached to the nano-carrier or be an entity of the nano carrier itself [55].

2.7.1.1. Liposomes functioning as nanocarriers, DC activating and targeting molecules

The manufacturer InvivoGen designed specific molecules to induce a potent immune response by activating different pathways triggered through PRR. Those molecules form positively charged liposomes, functioning as nano-carriers for nucleic acids such as dsDNA or mRNA. Those nano-carriers transport the respective nucleic acids into the cell where they allow the induction of the IRF pathway through activation of PRRs that recognize nucleic acids (e.g. the cytosolic DNA sensors DDX41 and IFI16 and the dsRNA receptors TLR3 and RIG-I/MDA-5). Those molecules also act as TLR2 and/or TLR7 agonists yielding an induction of the NF-κB pathway. TLR2 are expressed on the cell surface of many different immune cell types triggering the NF-kB pathway and thereby the production of pro-inflammatory cytokines, whereas TLR7 is an endosomal receptor predominantly found in DC and B cells that induces mainly the IRF pathway and the production of IFN-I.

Two of these liposome-forming molecules were selected for our experiments. One of the substances was CL419 (S-(2,3-bis(palmitoyloxy)-(2RS)propyl)-(R)-cysteinyl spermine) (fig. 6). The molecule was derived from Pam2CSK4 by replacement of Ser-(lys)4 by a cationic sperminyl group. Pam2CSK4 is a synthetic diacylated lipopeptide and a well-established TLR2 ligand. Accordingly, CL419 acts as TLR2 agonist forming positively charged liposomes allowing them to engulf negatively charged nucleic acids and to transport them into the nucleus inducing the NF-κB and IRF pathway. In vivo experiments suggest that intratumorally injected CL419, complexed with pDNA, yields a modest reduction of tumor growth [82].

Another molecule deriving from Pam2CSK4 is CL553 (N4-(S-((2,3-bis(palmitoyloxy))-(2RS)propyl)-(R)-cysteinyl) N1- (4- (((6- amino-2- (butyl amino) -8-hydroxy-9H-pur in-9-yl)methyl)benzoyl) glycinyl) spermine or PamadiFectin^{TM}) (fig. 7). CL553 was generated by adding CL307 (potent TLR7 agonist) to a Pam2C group. Therefore, CL553 functions as a dual TLR2 and TLR7 agonist inducing both the NF-κB pathway as well as the IRF pathway.

CL553 is also able to bind negatively charged nucleic acids yielding the induction of the IRF pathway. Experiments with B16-F1 tumor bearing mice resulted in a reduction of tumor growth and an improved longtime survival when CL553 complexed with tumor antigen-encoding pDNA was injected intratumorally [83].

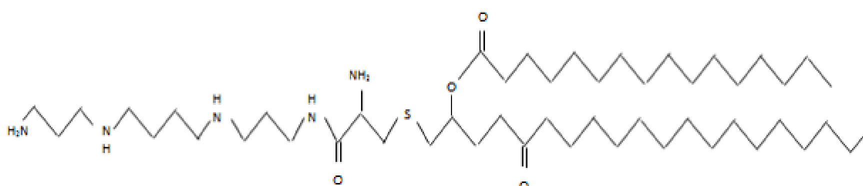


Fig. 6: Molecular structure of CL419

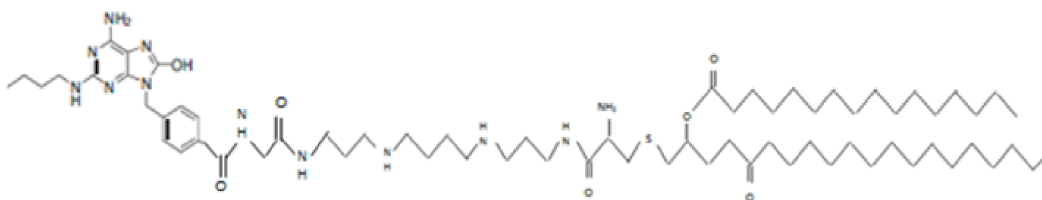


Fig. 7: Molecular structure of CL553 (PamadiFectin™)

2.7.1.2. Nucleic acids functioning as DC activating adjuvant

Two types of immunostimulatory nucleic acids were include in our trials, the first being synthetically produced CpG ODNs (oligodeoxynucleotides). CpG ODN are short synthetical single-stranded DNA molecules that contain high amounts of unmethylated CpG motifs. Unmethylated CpG motifs are considered PAMPs due to their high presence in the microbial genome compared to the vertebrate genome. CpG motifs are recognized by TLR9 present in the endosomal compartments of DC and B cells. Three different classes of CpG ODNs have been characterized due to their structure, function and effect on the immune response. Class A CpG ODNs yield a high IFN-I production, but only a weak TLR9 activation. Class B CpG ODNs strongly stimulate B cells and DC and activate TLR9-dependent NF-κB signaling. However, their impact on IFN-α secretion is weak. Examples for Class B CpG ODNs that were used in the experiment are ODN 1826 (also the internal control of our laboratory), ODN D-SL01 and ODN BW006. Class C CpG ODNs combine the characteristics of the classes A and B; they induce a high IFN-α production and yield a strong B cell stimulation. In our experiments we included ODN 2395, ODN M362 and ODN D-SL03 from the group of Class C CpG ODNs [84].

Secondly, double-stranded virus-derived DNA was included in the trials. Intracellular pathogen DNA is generally recognized by multiple CDSs. In our trials we included three different viral oligonucleotides (HSV-60, ISD and VACV-70). HSV-60 is a 60bp herpes simplex virus 1 genome derived ODN. HSV-60 is recognized by IFI16 and DDX41 and yields a STING-, TBK1- and IRF3-dependent IFN-β production. ISD (interferon stimulatory DNA) is a 45bp long oligonucleotide that derives from the Listeria monocytogenes genome. It also enhances the IFN- β production via the STING-, TBK1- and IRF3 pathway. VACV-70 is a 70bp long

from vaccinia virus genome deriving oligonucleotide. As well as HSV-60, VACV-70 is recognized by IFI16 and DDX41 and yields a STING-, TBK1- and IRF3-dependent IFN-β production [85].

2.7.1.3. Transfection reagents for testing nucleic acids

Two well established transfection agents were selected as internal controls.
JetPEI derives from linear polyethylenimine (PEI) and is a non-lipid transfection reagent. It facilitates intracellular DNA delivery by complexing DNA into positively charged particles that are capable to interact with cell surface molecules. By this, the complexes are endocytosed [86]. JetPEI has shown effective and reproducible DNA transfection and a low cytotoxicity in several cell lines [87].
The second transfection reagent used was DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate), designed by Sigma-Aldrich, representing one of the first generation transfection reagents. DOTAP is a cationic lipid, forming liposomes that can complex DNA (but also other negatively charged nucleic acids, e.g. RNA, oligonucleotides, and proteins). It is suited for in vitro as well as for in vivo applications in eukaryotic cells. The DOTAP/DNA complexes fuse with the cell membrane and the DNA is released into the cytosol. There are no cytotoxic effects observed [87].

2.7.1.4. Ovalbumin (OVA) functioning as tumor specific antigen

Ovalbumin is the main contributing protein to chicken egg white and a key reference protein for immunization. Due to its molecular structure is shows an immunogenic effect. Immunogenic OVA-derived peptides are efficiently presented by MHCII molecules on immature and mature DC. However, cross-presentation of OVA-protein on MHCI molecules was only observed in matured DC. Certain OVA-peptides yield a better T cell activation than OVA protein. For example, SIINFEKL (OVA 257-264) pulsed DC, provoke a stronger cytotoxic T cell response, than OVA-protein pulsed DC [88].

3. Material

3.1. Laboratory Devices

Device	Manufacture
Attune NxT acoustic focusing cytometer	ThermoFisher Scientific, Darmstadt
Centrifuge: Heraeus Megafuge 40R – _TX 1000 Rotor	ThermoFisher Scientific, Darmstadt
Centrifuge Sigma 1-14	Sigma Laborzentrifugen GmbH
CO2 Incubator	Binder, Tuttlingen
Light microscope Olympus CK2	Olympus
Microwave AEG Micromat	Electrolux Haushaltsgeräte
Vortex Mixer RS 2	IDLGmbG & Co. KG

3.2. Buffers and Solutions

Medium	Content
Dendritic cell (DC) medium	500 ml Iscove's Modified Dulbecco's Medium 5% FCS 100U/mL Penicillin/Streptomycin 2mM Glutamine 50µM ß-mercaptoethanol 10ng/mL GM-CSF
Wash medium	500 ml Eagle's Minimum Essential Medium 2% FCS 100U/mL Penicillin/Streptomycin 2mM Glutamine 50µM ß-mercaptoethanol
FACS medium	500ml PBS 2% FCS 100U/mL Penicillin/Streptomycin

	2mM EDTA

3.4. Expendable Materials

Material	**Manufacture**
Cell culture Flask, 25 cm2	Greiner Bio-One, Frickenhausen
Cell culture plate 6 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 12 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 96 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell strainer, 40µl	Falcon, Fisher Scientific, Schwerte
Eppendorf Safe-Lock Tubes 1.5 ml	Eppendorf
Greiner Tubes, 15 ml	Greiner-One, Frickenhausen
Greiner Tubes, 50 ml	Greiner-One, Frickenhausen
MACS Cell separation column MS	Miltenyi Biotec, Berg. Gladbach
MACS Cell separation column LS	Miltenyi Biotec, Berg. Gladbach
Neubauer Cell counting chamber	Superior, Marienfeld
Petridishes	Greiner Bio-One, Frickenhausen
Pipett tips, 0.1-10µl	Carl Roth, Karlsruhe
Pipett tips, 10-200µl	Carl Roth, Karlsruhe
Pipett tips, 100-1000µl	Carl Roth, Karlsruhe
Plastic pipetts 5ml,10ml,25ml	Cellstar, Germany
Round-bottom FACS tubes, 5ml	Corning GmbH, Wiesbaden
Syringe, 0.5 ml	Braun, Melsungen

3.5. Antibodies

Antibody specificity	**Fluorescence label**	**Manufacture**
CD11c	APC	eBioscience
CD80	PE	eBioscience
CD86	PE Cy7	eBioscience
MHCII	e-Flour450	eBioscience

3.6. Reagents and chemicals

Substance	Manufacture
Ethanol 70%	Brüggemann, Heilbronn
CpG 1826	Sigma-Aldrich
CL419	InvivoGen
CL553 (PamadiFectin™)	InvivoGen
DOTAP Liposomal transfection agent	Sigma-Aldrich
LPS	Sigma-Aldrich
Dulbecco's phosphate buffered saline	Sigma-Aldrich
Isocove's modified dulbecco's medium	Sigma-Aldrich
EDTA (Ethylendiamintetraacetat)	Sigma-Aldrich
FACS Clean Solution	BD Pharmingen, Heidelberg
FACS Flow Sheath fluid	BD Pharmingen, Heidelberg
FACS Rinse Solution	BD Pharmingen, Heidelberg
FCS, fetal calf serum	PAA Laboratories, Cölbe
Forene (Isoflurane)	Abbott, Wiesbaden
GM-CSF	R&D, Wiesbaden
jetPEI	Polyplus transfection
L-Glutamin	Gibco Life Technologies
ß-Mercaptoethanol, 99%p.a.	Carl Roth, Karlsruhe
Ovalbumin	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Trypanblau solution	Sigma-Aldrich

3.7. Oligonucleotides

Oligonucleotide	Sequence (5'->3')	Manutacture

HSV60-s	TAAGACACGATGCGATAAAATCTGTTTGTAAAA TTTATTAAGGGTACAAATTGCCCTAGC (60)	Eurofins Genomics
HSV60-as	GCTAGGGCAATTTGTACCCTTAATAAATTTTACA AACAGATTTTATCGCATCGTGTCTTA (60)	Eurofins Genomics
ISD-s	TACAGATCTACTAGTGATCTATGACTGATCTGTAC ATGATCTACA (45)	Eurofins Genomics
ISD-as	TGTAGATCATGTACAGATCAGTCATAGATCACTAG TAGATCTGTA (45)	Eurofins Genomics
VACV-70-s	CCATCAGAAAGAGGTTTAATATTTTTGTGAGACCA TCGAAGAGAGAAAGAGATAAAACTTTTTTACGACT	Eurofins Genomics
VACV-70-as	AGTCGTAAAAAAGTTTTATCTCTTTCTCTCTTCGAT GGTCTCACAAAAATATTAAACCTCTTTCTGATGG	Eurofins Genomics
ODN D-SL01	TCGCGACGTTCGCCCGACGTTCGGTA	Eurofins Genomics
ODN D-SL03	TCGCGAACGTTCGCCGCGTTCGAACGCGG	Eurofins Genomics
ODN M362	TCGTCGTCGTTC:GAACGACGTTGAT	Eurofins Genomics
ODN 2395	TCGTCGTTTTCGGCGC:GCGCCG	Eurofins Genomics
ODN BW006	TCGACGTTCGTCGTTCGTCGTTC	Eurofins Genomics

3.8. Cell Culture

Bone marrow cells (BM-DC) were cultured at 37°C and 5% CO2 in IMDM media, supplemented with fetal calf serum (5%), glycine (1%), penicillin/streptomycin (1%), ß-mercaptoethanol (1%) and 10ng/ml GM-CSF.

3.9. Mice

C57BL/6J is one of the most widely used inbred strains of laboratory mice. C57Bl/6J mice were bread and kept by the Translational Animal Research Center (TARC) of the Johannes Gutenberg-Universität Mainz in accordance with the animal protection law (German TierSchG)

and the German Tierschutz-Versuchstierverordnung (TierSchVersV). All mice were kept in a controlled microbial environment and used at the age of 8-12 weeks.

3.10. TLR-2 -/- Mice

TLR2-deficient mice on C57BL/6 background (B6.129-Tlr2^{tm1Kir}/J) were obtained from the lab of Prof. Kerstin Steinbrink (Dept. of Dermatology, University Medical Center Mainz).

4. Methods

4.1. Obtaining murine bone marrow-derived dendritic cells (BMDC)

WT and TLR2-deficient BL6 mice were used to investigate DC activation after treatment with different immunoactive substances.

The mice were killed by cervical dislocation. The os coxae, the femurs and the tibiae were dissected in order to obtain the bone marrow cells from those bones by flushing them with wash medium. They were then spun down in the centrifuge and resuspended with 2ml of Grey's lysis in order to deplete erythrocytes. After 1 min the lysis was stopped by adding wash medium. The cell suspension was again spun down and resuspended in 10ml wash medium. The cells were diluted 1:10 with Trypanblau solution. Then they were counted under the light microscope by means of a counting chamber. Cells were then diluted with BMDC medium in an order to attain 200.000 cells/ml. They were then distributed in 12 well cell culture plates (1ml in each well9 and put in the CO2-incubator for seven to eight days. On day three and six medium was replenished.

4.2. BMDC activation assays with liposomes and oligonucleotides

BMDS differentiated in 12-well plates were treated with different substances. LPS (5mg/ml, 1:10 in distilled water, 2µl) and untreated cells were used as controls in all experiments. Also, single staining of all fluorescent-labeled antibody (APC-conjugated anti-CD11c-antibody, PE-conjugated anti-CD80-antibody, PE Cy7-conjugated anti-CD86-antibody, eFL450-conjugated anti-MHCII-antibody) and an unstained control were prepared for FACS analysis.

After 24 hours the cells were harvested and used for FACS assays.

4.2.1. Treatment of BMDC (WT) with TLR2-targeting liposomes CL419/CL553

CL419/CL553 were resuspended at a concentration of 1mg/ml. BMDC were treated with five different exponentially increasing quantities (100pg/ml; 1ng/ml; 10ng/ml; 100ng/ml; 1µg/ml) of CL419 and CL553.

4.2.2. Treatment of BMDC with synthetic ODN in a soluble form or in combination with the transfection agent jetPEI

Different double-stranded synthetic oligonucleotides (ODN D-SL01, ODN BW006, ODN 2395, ODN M32, ODN D-SL03) were diluted in endotoxin-free water (1µg/µl) and then put in 65°C warm water for 10 min. Afterwards, they were put on ice for 5 min. BMDC were treated with a low (100ng/ml) and a high (1µg/ml) concentration of ODN.
In parallel settings, ODN were complexed with the transfection agent jetPEI. To this end, 3 µl of jetPEI was diluted with 150 µl NaCl. 1,5µg of the different ODN where diluted in NaCl and then added and incubated for 20 min at 4°C. Then, the complexed ODN then were used at the same final concentrations (100 ng/ml, 1 µg/ml) to treat BMDC.

4.2.3. Treatment of BMDC (WT) with synthetic ODN in a soluble form or in combination with the transfection agent DOTAP

The experiments were continued with the best performing ODN's (ODN D-SL01 and ODN D-SL03) from the prior trial (4.2.2.). They were prepared as explained before. Additionally, an internal control (ODN 1826) was used. ODN 1826 (500ng/µl) was diluted in PBS, the other ODN's were diluted as explained before (4.2.2).
Considering the results from the prior trial (4.2.2.), quantities between 50ng/ml to 500ng/ml were used (50ng/ml<250ng/ml<500ng/ml). Accordingly, the different ODN's were applied in a soluble form. Since the transfection agent jetPEI had no considerable effect on the activation of BMDC in comparison to the soluble ODN's, a different transfection agent (DOTAP) was chosen for further trials. Therefore, DOTAP was diluted 6,4:50 with serum-free medium. 1µg of each ODN was diluted in 50 µl serum-free medium. Then, Pre-diluted DOTAP and ODN were mixed an incubated for 15 min at RT. BMDC were treated using complexed ODN (50ng/ml<250ng/ml<500ng/ml). After 3 hours 25µl of FCS was added to each well.

4.2.4. Treatment of BMDC (WT) with viral oligonucleotides in a soluble form or in combination with the transfection agent DOTAP

Each 50µl of the sense and anti-sense strand of each viral oligonucleotides (HSV-60, ISD, VACV-70) were mixed in a 2 ml safe lock tube, diluted with endotoxin-free water to 1µg/µl.

The reagents then were annealed in boiling water (100°C) for 10 min and let cool down at RT afterwards.
The cells were stimulated with different quantities of the soluble oligonucleotides (0.1µg/ml<1µg/ml).
Next, the oligonucleotides were complexed with the transfection agent DOTAP. For this purpose, DOTAP was diluted 6,4:50 with serum-free medium. 1µl of each oligonucleotide was diluted in 50 µl serum-free medium. Then the two reagents were mixed an incubated for 15 min at RT. Next the BMDC were stimulated by the complexed oligonucleotides in the same concentration as before (0.1µg/ml<1µg/ml). After 3 hours 25µl of FCS was added into each well.

4.2.5. Treatment of BMDC (WT and TLR2-deficient mice) with nucleic acids complexed with CL419/CL553

The previously used nucleic acids (ODN 1826, ODND-SL001, ODND-SL03 and HSV-60, IDN, VACV-70) were selected for complexation with CL419 and CL553. For direct comparison trials with nucleic acids complexed with DOTAP were performed simultaneously (as in 4.1.2.3./4.1.2.4.).

CL419/CL553 was diluted 1:100 in PBS and incubated for 10 min at RT.
For complexation CL419 and CL553 were used in concentrations where 50 % of the highest activation of BMDC could be yielded in previous trials (10ng/ml see tables 1-3).
The recommended ratio (by the manufacturer InvivoGen) from CL419/CL553 to nucleic acid is 63,82ng:1µg. Accordingly, 167ng nucleic acid were complexed with 10ng CL419/CL553.

First trials included different synthetically generated ODN’s (ODN 1826, ODND-SL001, ODND-SL03) that were transfected with diluted CL419/CL553. For complexation they were incubated for 20 min at RT.
Only one concentration of CL419/CL553 (10ng/ml) was used either complexed with ODN or in a soluble form. Also, the ODN’s were solubly tested (167ng/ml<1µg/ml).
Trials with complexed ODN’s and DOTAP were executed simultaneously in order to obtain comparison parameters. Hereby, concentrations of 167ng/ml<1µg/ml were used for the ODN’s. 167ng/ml were chosen for reasons of better comparability as well as 1µg/ml, which has been a standard dose for trials performed with complexed ODN’s with DOTAP.

Secondly, different viral oligonucleotides (HSV-60, ISD, VACV-70) were transfected with CL419/CL553 as described above and incubated for 20 min at RT.
Dosages of 10ng/ml CL419/CL553 either complexed with ODN or in a soluble form were used in the trial. Also, the viral oligonucleotides were tested alone (167ng/ml<1µg/ml).
Viral oligonucleotides complexed with DOTAP have been used in the trials as comparison parameters. Quantities for the viral oligonucleotides were chosen as above (167ng/ml<1µg/ml).

In further trials CpG 1826 and HSV-60 being the best performing nucleic acids from prior results have been selected for further investigations.
CpG 1826 (500ng/µl) and HSV-60 (1µg/ml) were diluted in PBS. Accordingly, 167ng of each substance was transfected with CL419/CL553 (10ng/ml) and incubated for 20 min at RT.
In this trial different quantities of the complexed CL419/CL553 (5ng<10ng<25ng) and nucleic acids (the quantity of the nucleic acid hereby depending on the quantity of CL419/CL553) were used. CL419/CL553 were also tested in a soluble, not complexed form at the same concentrations. Also, the nucleic acids were tested alone (4,2ng/ml).

The same trial including CpG 1826 and HSV-60 was performed on TLR2 deficient cells in comparison to WT cells, only using a quantity of 25ng for CL419/CL553 and the respective amount of each nucleic acid.

4.2.6. Staining of the treated BMDC with fluorescent labeled antibodies against surface markers of activated DC

Within and on the surface of DC there are certain proteins that can be labeled with fluorescent antibodies. For staining and analysis stimulated BMDC were washed with FACS medium. Free Fc-receptors were blocked with anti-mouse Fc-antibody 2.4G2 (anti-CD16/32; 1 µg/10^6 cells in 100 µl) for 15 min at RT. After that the cells were stained with suitable fluorescent antibodies in a concentration between 0.2 and 0.5µl/ml. The fluorescent antibodies used for analysis were APC-conjugated anti-CD11c-antibody, PE-conjugated anti-CD80-antibody, PE Cy7-conjugated anti-CD86-antibody, eFL450-conjugated anti-MHCII-antibody. They were incubated for 20 min at 4°C. Afterwards the cells were washed with FACS medium and fixed with PFA (Paraformaldehyde, 0.5-4% in PBS) and then transferred to 5ml FACS tubes.

4.3. Flow Cytometry

The flow Cytometry is a device used to measure, count and analyze cells regarding their size, internal complexity/granulation and relative fluorescence intensity. Therefore, the single separated cells pass laser beams whereby the cells scatter the light and emit fluorescence.
The forward-scattered light (FSC) is proportional to the cell size whereas the sideward-scattered light is proportional to the cell's internal complexity or granulation. Fluorescence is emitted by cells that had been primarily stained with fluorescent antibodies.
Any cells within a diameter range between 0.2 to 150 µm are suitable for analyzation.

The cytometer consists of three main units: Fluids, optics and electronics. The fluid unit is used for cell transportation. The optic unit contains the lasers, optical filters and detectors. The electronics convert the detected light signals into electronic information that can be processed by a computer, whereupon the data can be analyzed.

4.3.1. Fluorescent labeled Antibodies used in FACS analysis

The antibodies that were used were APC-conjugated anti-CD11c-antibody, PE-conjugated anti-CD80-antibody, PE Cy7-conjugated anti-CD86-antibody and eFL450-conjugated anti-MHCII-antibody. To investigate the activation of BMDC a FACS analysis was performed. The intensity of the detected light signal correlates with the quantity of surface markers that is expressed on activated DC. Hence its intensity correlates with the degree that each DC has been activated. The measured fluorescent emission the activated cells have been statistically evaluated and are presented as MFI's (mean fluorescent intensities).

4.3.2. FACS analysis of the fluorescent labeled BMDC

For FACS analysis the "Attune NxT acoustic focusing cytometer" from ThermoFisher Scientific with the correspondent Software "Attune Nxt Flow Cytometer Software – Version 3.1" was used.
The gating strategies used for analyzation can be found in the appendix.

5. Results

The results were presented as mean fluorescence intensities (MFI) of fluorescence labeled surface markers on DCs. The fluorescence activity of one cell is proportional to the number of surface markers expressed on it. The MFI was not depicted as an absolute number because of the interindividual differences. It was instead depicted as a multiple of a control. As a control we chose an untreated cell sample, in other words cells that were not intentionally activated by any substance.

5.1. Gating strategy

In the following figure (fig. 8) there is a representation of the gating strategy used for FACS analysis, gating all viable cells in a first step, sorting out the duplets in a second step and third specifying CD11c positive cells with a high expression of MHCII.

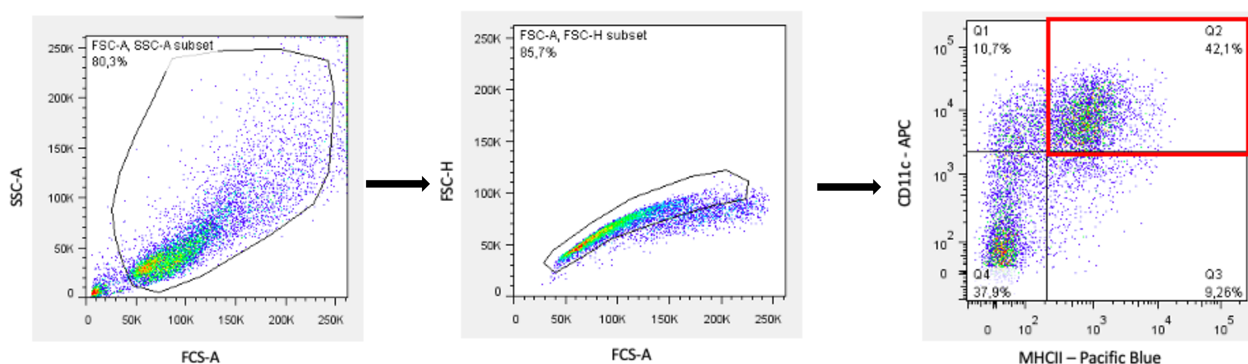


Fig. 8: Gating strategy for BMDC in FACS analysis: In the first step all viable cells were gated plotting SSC-A versus FCS-A. In the second step all cell duplets are sorted out by plotting FSC-H versus FCS-A. In the third step, only CD11c positive cells (e.g. DC) that showed a high expression of MHCII are selected.

The figure below (fig. 9) shows an example of the primary FACS data, depicting the fluorescence intensities of different surface markers on untreated BMDC, BMDC stimulated with LPS and BMDC treated with TLR2 targeting liposomes. The untreated cells show a low intensity compared to the BMDC stimulated with LPS. BMDC treated with CL419 or CL553 also show a higher fluorescence intensity than untreated cells.

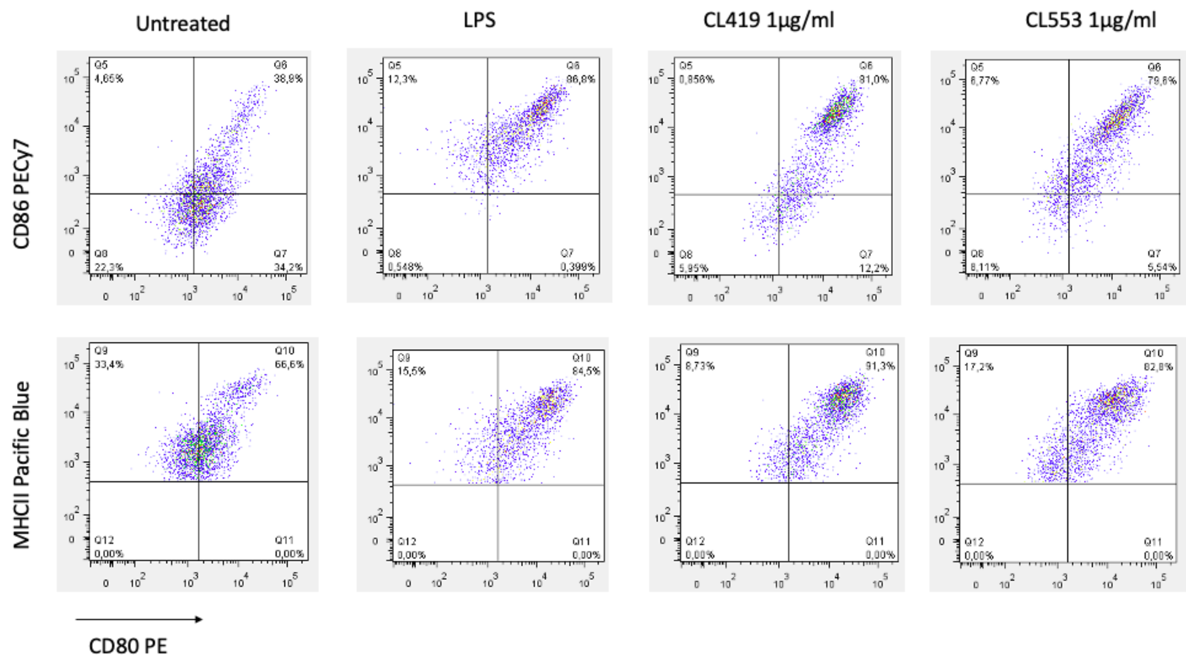


Fig. 9: Example of primary FACS data: measurement of fluorescence intensities of different surface markers expressed on BMDC that were either untreated (resulting in a low expression of surface markers) or treated with LPS (resulting in a high expression of surface markers) compared to BDMC treated with CL419 or CL553.

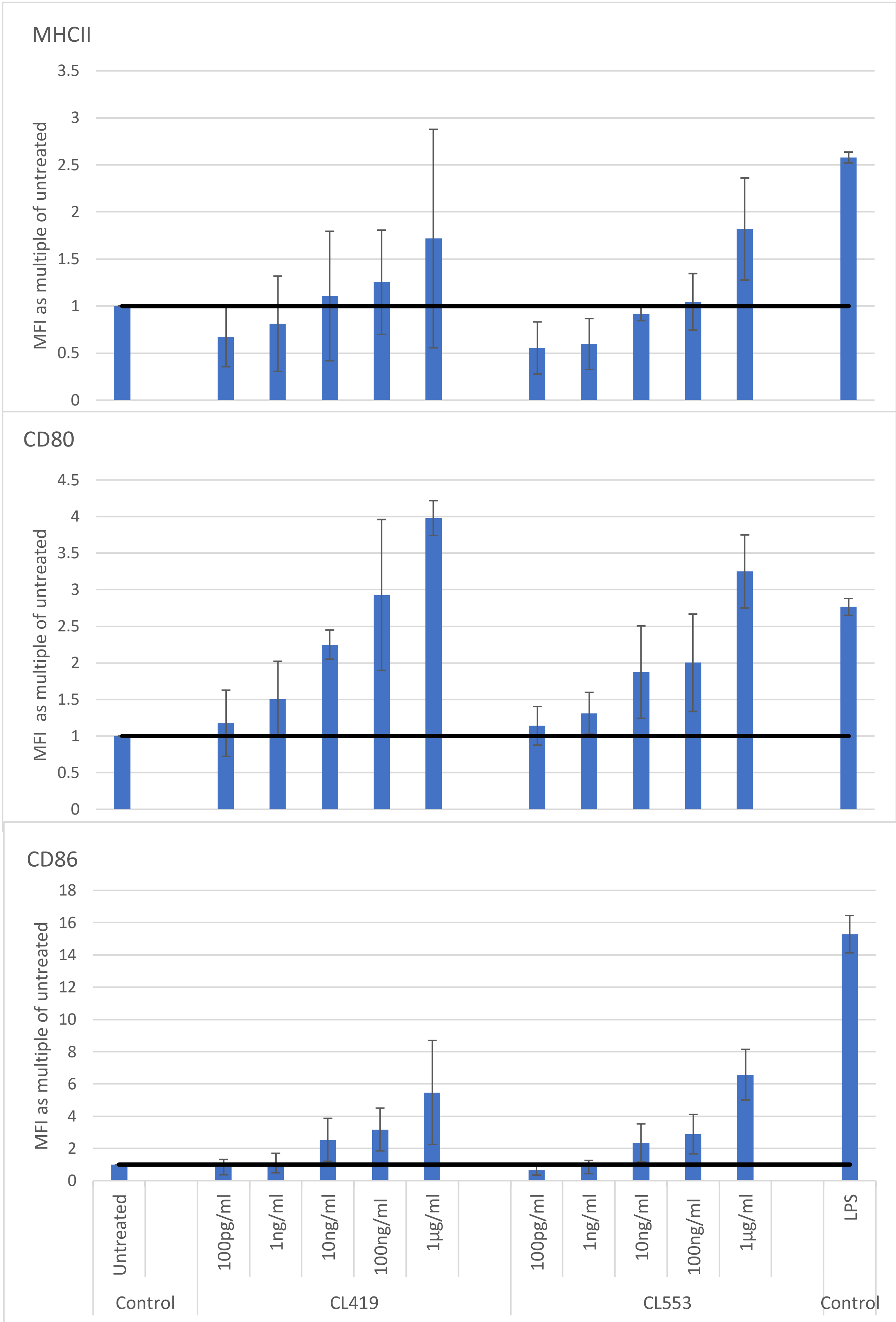
5.2. Activation of BMCD by TLR2-targeting liposomes

The activation of BMDC by TLR2-targeting liposomes (CL419/CL553) was tested using different doses of either liposome. BMCD differentiated from bone marrow cells of B6 mice (n=3) were treated with different concentrations of CL419/CL553 as previously explained. After 24 hours of incubation the cells were stained with fluorescent-labeled antibodies that target general differentiation markers of DC (CD11c) and surface markers of activated DC (CD80, CD86, MHCII). The results of the FACS analysis are depicted in figure 10. The figures depict the respective means and SD of the mean fluorescence intensity (MFI) signals of the different fluorescent-labeled surface marker (CD80, CD86, MHCII) detecting antibodies. LPS

was used as a positive control. Also, an untreated sample was included. The numbers are given as multiple of the untreated control to minimize inter-individual differences.

The upregulation of CD80 was higher compared to the LPS control, when treated with CL419 in a quantity of 100ng and 1µg and for CL553 in a quantity of 1µg. CD86 and MHCII upregulation was about half as high compared to the LPS control.

The tables show a dose-dependent increase of the signal intensities, especially regarding CD80 and CD86. The surface markers CD80 and CD86 were significantly increased using concentrations of 10 ng/ml and higher. MHC II only showed a significant upregulation for the highest concentration of CL553, which is due to a high standard deviation (SD). A high standard deviation indicates that the cells of the individual mice reacted quite differently to the liposomes. In the BMDC of some individuals the liposomes showed a high upregulation of MHCII, others did not react with such a strong upregulation. Why the cells of the different individuals reacted to differently in regards of MHCII upregulation is to be further investigated.



MHCII
MFI as multiple of untreated
3.5
3
2.5
2
1.5
1
0.5
0
CD80
MFI as multiple of untreated
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CD86
MFI as multiple of untreated
18
16
14
12
10
8
6
4
2
0
Untreated
100pg/ml
1ng/ml
10ng/ml
100ng/ml
1µg/ml
100pg/ml
1ng/ml
10ng/ml
100ng/ml
1µg/ml
LPS
Control
CL419
CL553
Control

Fig. 10: BMCD treated with CL419/CL553: Dose dependent upregulation of MHCII, CD80, CD86.

5.3. Activation of BMDC by ODNs in a soluble form and complexed with jetPEI or DOTAP

The activation of BMDC by ODNs was tested using different doses of either liposome. BMCD isolated from the bones of B6 mice were treated with different concentrations of ODN's either in a soluble form or complexed with transfection agents as previously explained (4.1.2.2.; 4.1.2.3.).
The preparation and performance of the FACS analysis was performed as described before (5.1.).
The results of the FACS analysis are depicted in fig. 11 and fig. Appendix 2. Those figures depict the respective means and SD of the intensities of the signals deriving from the different fluorescent-labeled surface markers. LPS was used as a positive control in all cases. Also, an untreated negative control in included in all tables. The transfection agents themselves was included in the trial as a control in order to assess their potential stimulatory activity. As previously, the numbers are depicted as multiple of the negative control.
Only one experiment (n=1) was performed with ODN's complexed with jetPEI (as in 4.1.2.2.). In this experiment we used five different ODN's (ODN D-SL01, ODN D-SL03, ODN BW006, ODN 2395, ODN M32).
When complexed with jetPEI no significant increase in the activation of DC, measured by the expression of surface markers, could be yielded for any concentration of any ODN. ODN D-SL01 and ODN D-SL03 yielded an upregulation of surface markers similar to LPS when used alone and in a high concentration. In consequence no further trials with the transfection agent jetPEI were conducted. This could be the result of an insufficient complexation due to the protocol or instable complex formation or the lack of endosomal release. (Fig. Appendix 2)
We repeated the trials with soluble ODN's and chose those that induced the highest activation (fig. 11) for further trials (n=4) with DOTAP as a transfection agent (as in 4.1.2.3.).
For the trials performed using the transfection agent DOTAP, ODN 1826 was included as internal standard. For all of the three included ODN's an increase of signal intensity proportional to its concentration could be observed. Also, the intensity increased when using DOTAP as a transfection agent. The highest activation could be reached with ODN 1826 complexed with DOTAP concentrated at 500ng/ml.

In a soluble form some of the ODNs were, in a high concentration (1µg/ml), able to yield an increase of intensity. Those ODNs included ODN D-SL01 and ODN D-SL03. In conclusion, those ODNs do not just cause an activation of BMDC via TRL9, but also seem to have a targeting property.
The transfection reagent DOTAP was used for further trials for complexation with the two ODNs that yielded a BMDC activation when used in a soluble form. Also, ODN 1826 was additionally included in the trials, being a well-established ODN in our laboratory.
CD80 upregulation was significant for all ODNs at all concentrations in a soluble as well as in a complexed form with DOTAP. The CD80 upregulation was higher (max. threefold) compared to the LPS control. CD86 was significantly upregulated when ODN 1826 and ODN D-SL03 were complexed with DOTAP even at the lowest (50ng/ml) concentration. In case of complexed ODN D-SL01/DOTAP CD86 was significantly upregulated at 250ng/ml and higher concentrations. When applied directly, the ODNs where only able to yield a significant upregulation in the highest concentration applied (ODN 1826, ODN D-SL01) or not at all (ODN D-SL03). An upregulation similar or modestly higher compared to the LPS control was achieved for each ODN at the highest quantity complexed with DOTAP.
MHCII upregulation was significant for ODN/DOTAP complexes at the highest concentration (500ng/ml) (and 250ng/ml for ODN 1826). In a soluble form none of the ODNs could yield a significant upregulation. The upregulation by the ODN and ODN/DOTAP complexes was in every combination similar or higher (max. 1,5-fold) compared to the LPS control.

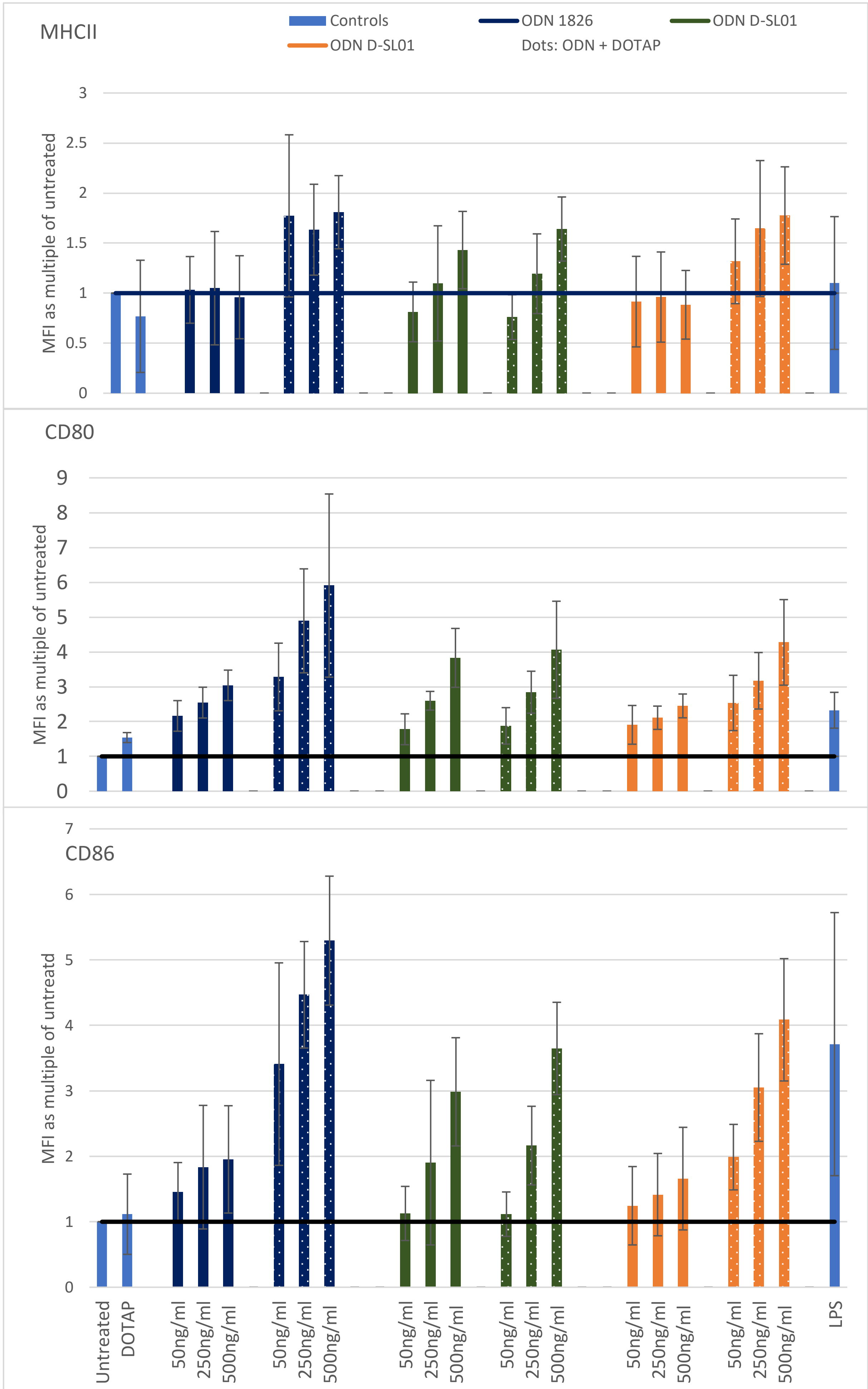

MHCII
Controls
ODN 1826
ODN D-SL01
ODN D-SL01
Dots: ODN + DOTAP
MFI as multiple of untreated
3
2.5
2
1.5
1
0.5
0
CD80
MFI as multiple of untreated
9
8
7
6
5
4
3
2
1
0
7
CD86
MFI as multiple of untreatd
6
5
4
3
2
1
0
Untreated
DOTAP
50ng/ml
250ng/ml
500ng/ml
50ng/ml
250ng/ml
500ng/ml
50ng/ml
250ng/ml
500ng/ml
50ng/ml
250ng/ml
500ng/ml
50ng/ml
250ng/ml
500ng/ml
50ng/ml
250ng/ml
500ng/ml
LPS

Fig. 11: BMCD treated with ODN D-SL01 (dark green), ODN D-SL03 (orange) and ODN 1826 (dark blue) in a soluble form and complexed with DOTAP (dotted bars): upregulation of MHCII, CD80, CD86.

5.4. Activation of BMDC by virus-derived ODN

The activation of BMDC by virus-derived ODN oligonucleotides (ISD, HSV-60, VACV-70) was tested using different doses of either liposome. BMCD isolated from the bones of B6 mice (n=3) were treated with different concentrations of soluble and complexed with DOTAP as previously explained (4.1.2.4.)
The results are depicted in figure 12. Neither ISD nor VACV-70 led to a significant activation of the cells, even when complexed with DOTAP. Only for HSV-60 complexed with DOTAP at a high quantity a moderately significant activation could be observed. To rule our errors in processing and storage a new stock of viral oligonucleotides was ordered and used in further trials for complexation with DOTAP and CL419/CL553. In comparison, the LPS control yielded a notable upregulation of all three surface markers (at least threefold).

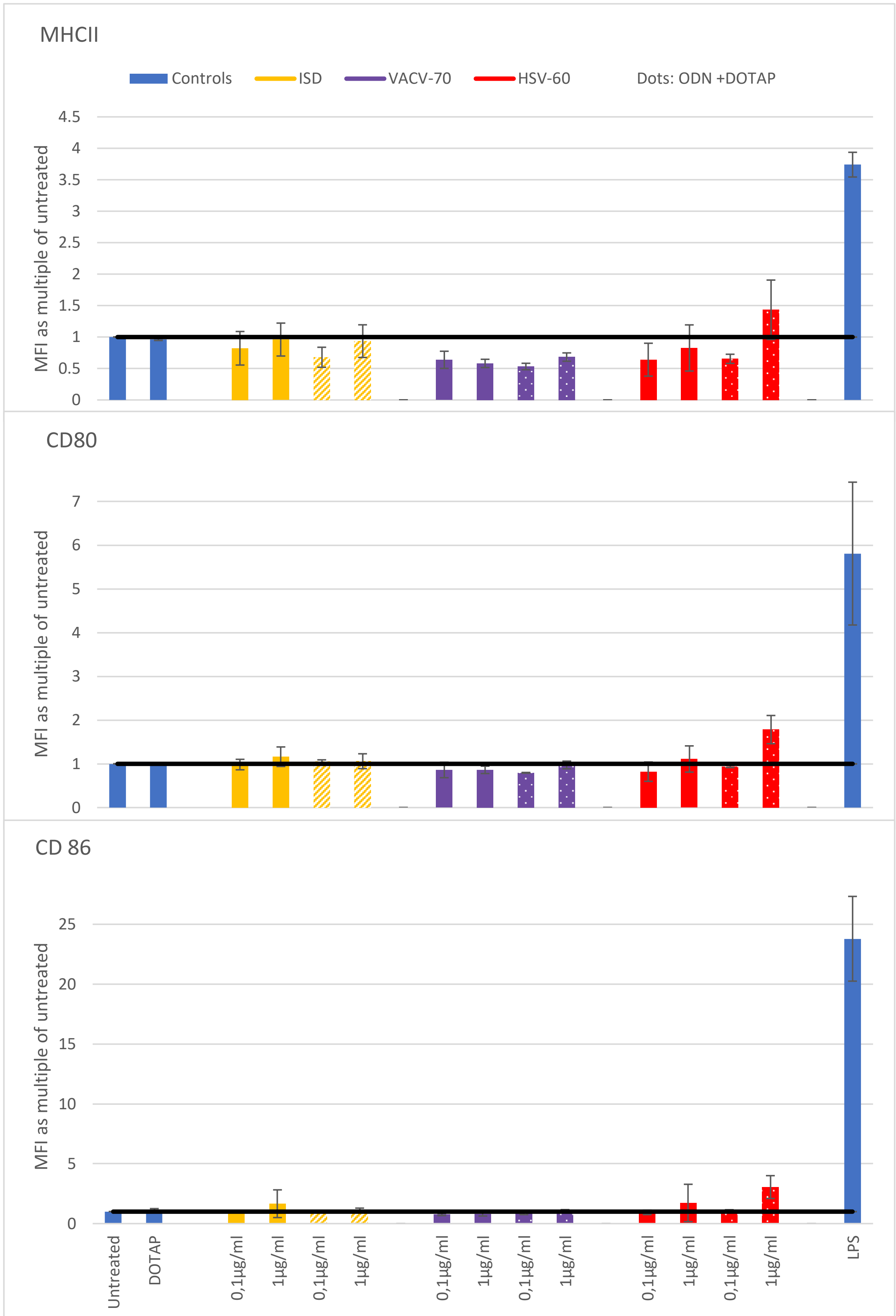

MHCII
Controls
ISD
VACV-70
HSV-60
Dots: ODN +DOTAP
MFI as multiple of untreated
CD80
MFI as multiple of untreated
CD 86
MFI as multiple of untreated
Untreated
DOTAP
0,1µg/ml
1µg/ml
0,1µg/ml
1µg/ml
0,1µg/ml
1µg/ml
0,1µg/ml
1µg/ml
0,1µg/ml
1µg/ml
0,1µg/ml
1µg/ml
LPS

Fig. 12: BMCD treated with ISD (dark yellow), VACV-70 (violet), HSV-60 (red) in a soluble form and complexed with DOTAP (dotted bar): upregulation of MHCII, CD80, CD86.

5.5. Activation of BMDC with ODN and virus-derived oligonucleotides complexed with CL419/CL552

Next, the activation of BMDC by distinct nucleic acids complexed with a TRL2-targeting liposome (CL419/CL553).

5.5.1. Activation of BMDC with ODN's complexed with CL419/CL552

In a soluble form all of the ODNs (ODN D-SL01, ODN D-SL03, ODN 1826) could yield an upregulation of the three surface markers CD80, CD 86 and MHCII at a high concentration (1µg/ml), as we had observed before. Only ODN 1826 in a soluble form could not increase MCHII significantly. When complexed with DOTAP all of the ODNs caused a significant upregulation of surface markers in BMDC from a low concentration (167ng/ml) and higher. The effect was moderately lower compared to the LPS control for CD80 and similar or moderately higher for CD86 and MHCII. Unfortunately, this effect could not be observed when complexed with CL419 or CL553. Only ODN 1826 complexed with CL419 could yield a significant upregulation of CD80, CD86 and MHCII (fig. 13).
CL419 alone in a concentration of 10ng/ml caused a modest but still significant upregulation of CD80 and CD86. Neither CL553 (in a concentration of 10ng/ml) nor DOTAP were able to yield any significant upregulation of surface markers. Considering the results from previous trials (5.1.), in which the liposomes were applied in a concentration in which their yielded a half-maximal effect it is not surprising, that the effect of CL553 in this trial had not been significant. So concludingly, the effect of CL419 and CL553 on BMDC could be reproduced in those trials. The highest activation of BMDC by ODN was yielded when ODNs were complexed with DOTAP. The best performing ODN in that case was ODN 1826 at the highest concentration used.

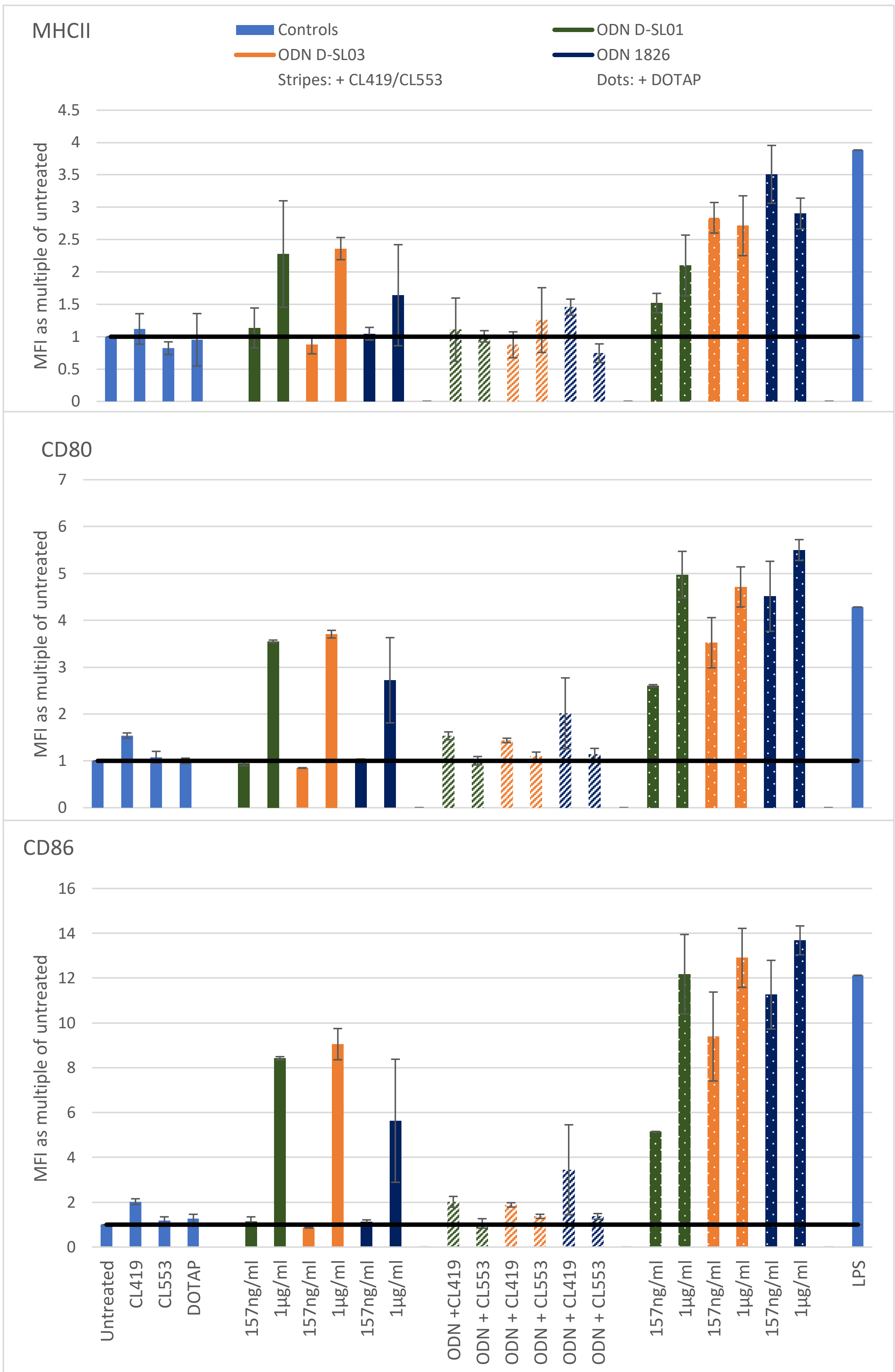

MHCII
Controls
ODN D-SL01
ODN D-SL03
ODN 1826
Stripes: + CL419/CL553
Dots: + DOTAP
MFI as multiple of untreated
CD80
MFI as multiple of untreated
CD86
MFI as multiple of untreated
Untreated
CL419
CL553
DOTAP
157ng/ml
1µg/ml
157ng/ml
1µg/ml
157ng/ml
1µg/ml
ODN +CL419
ODN + CL553
ODN + CL419
ODN + CL553
ODN + CL419
ODN + CL553
157ng/ml
1µg/ml
157ng/ml
1µg/ml
157ng/ml
1µg/ml
LPS

Fig. 13: BMCD treated with ODNs (ODN D-SL01 (dark green), ODN D-SL03 (orange), ODN 1826 (dark blue) in a soluble form and complexed with DOTAP (dotted bars) or CL419/CL553 (striped bars): upregulation of MHCII, CD80, CD86.

5.5.2. Activation of BMDC with viral oligonucleotides complexed with CL419/CL552

Secondly, the viral oligonucleotides (ISD, HSV-60 and VACV-70) from a new stock were used for trials (n=1) in complexes with CL419 and CL553 (as described in 4.1.2.5.). The highest upregulation of surface markers could be observed for HSV-60 in complexes with DOTAP. A modest upregulation of all surface markers could also be observed CL419 in complexes with HSV-60 and VACV-70. The effect of LPS was about twofold higher for CD80 and MHCII upregulation and notably higher for CD86, with the exception of HSV-60/DOTAP complexes. Here, LPS only showed a modestly higher upregulation.
CL419 in a concentration of 10ng/ml yielded a modest upregulation of surface markers as it could be expected regarding the results from prior trials. CL553 alone also yielded a high upregulation of all surface markers which had not been observed priorly. In prior trials were the dose dependent activation of Cl419 and CL553 was tested, CL553 had also yielded a significant upregulation of surface markers, so a significant activation of a certain quantity of BMDC could be expected. Nevertheless, the upregulation of CD80 and MHCII was comparably high and therefore could also trace back to an error in processing. (Fig. Appendix 3)

5.5. Activation of BMDC with the best performing nucleic acids complexed with CL419/CL552

On account of these results ODN 1826 and HSV-60 were chosen for complexation with the TLR2-targeting liposomes (n=6).
HSV-60 and ODN 1826 were tested alone in the same concentration (4,2ng/ml) in which they were used for complexation with CL419 and CL553. These nucleic acids alone did yield only a little increase of surface markers (MHCII) or no significant increase at all (CD80, CD86).
CL419 and CL553 alone were as before able to yield a modest but significant upregulation of CD80 and CD86 as well as CL419 increased the MHCII expression on BMDC.
When BMDC were treated with complexes of nucleic acids and TLR-targeting liposomes there occurred a synergistic effect on upregulation of surface markers on BMDC caused by the

simultaneously activation of BMDC through different pathways. All combination of nucleic acid and liposomes yielded a significant upregulation of CD80, CD86 and MHCII when used in a quantity of 2,5µl, some already in a concentration of 1µl. LPS yielded a higher upregulation of CD80 and CD86 compared to the oligonucleotides in complexes at a high concentration (max threefold higher). The MHCII upregulation of HSV-60 in complex with CL419/CL553 was similar to the LPS control (fig. 14).

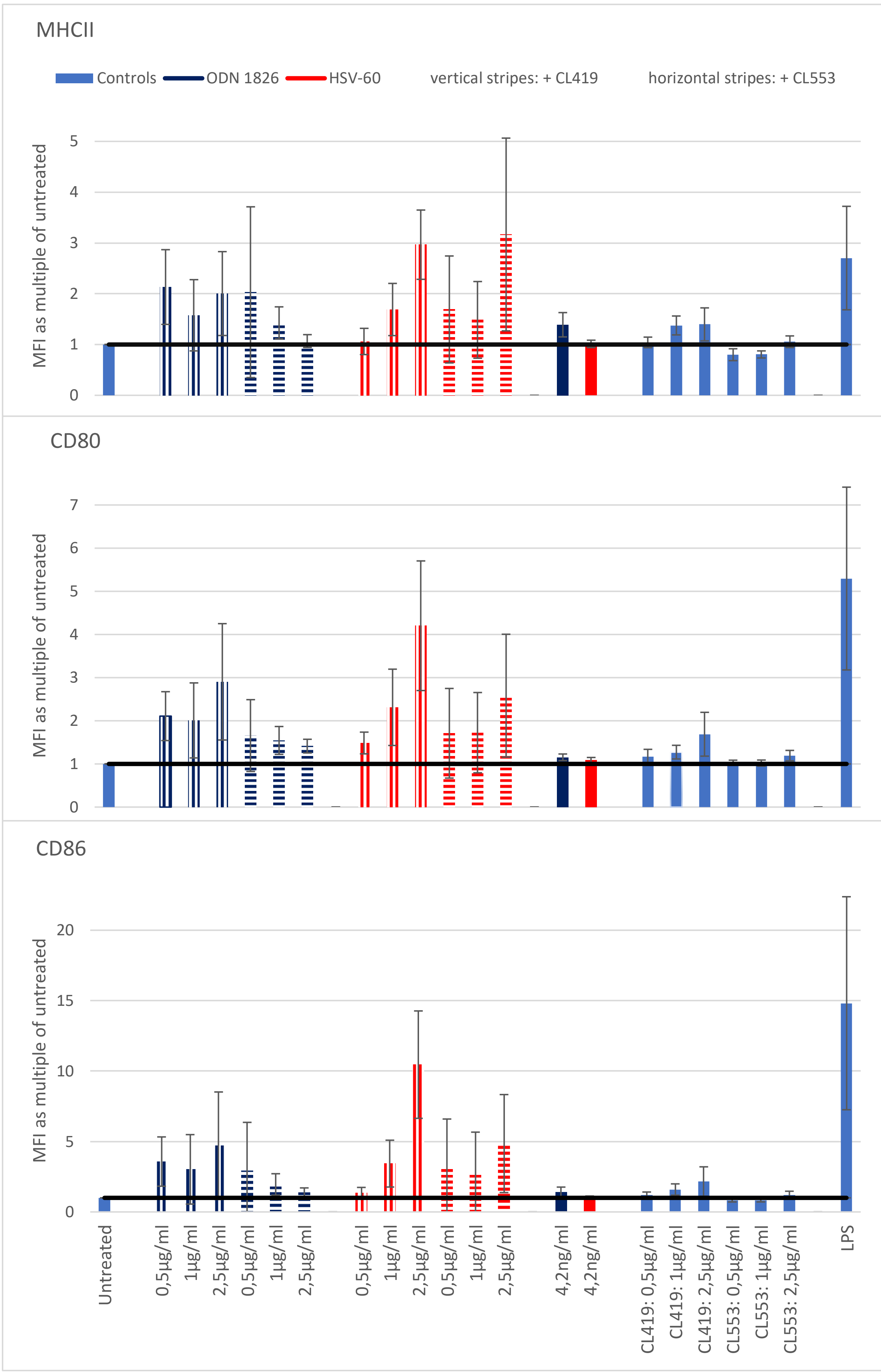

MHCII
Controls
ODN 1826
HSV-60
vertical stripes: + CL419
horizontal stripes: + CL553
MFI as multiple of untreated
CD80
MFI as multiple of untreated
CD86
MFI as multiple of untreated
Untreated
0,5µg/ml
1µg/ml
2,5µg/ml
0,5µg/ml
1µg/ml
2,5µg/ml
0,5µg/ml
1µg/ml
2,5µg/ml
0,5µg/ml
1µg/ml
2,5µg/ml
4,2ng/ml
4,2ng/ml
CL419: 0,5µg/ml
CL419: 1µg/ml
CL419: 2,5µg/ml
CL553: 0,5µg/ml
CL553: 1µg/ml
CL553: 2,5µg/ml
LPS

Fig. 14: BMCD treated with the best performing nucleic acids (ODN 1826 (dark blue), HSV-60 (red)) complexed with CL419 (vertically striped bars)/CL553 (horizontally striped bars): upregulation of MHCII, CD80, CD86

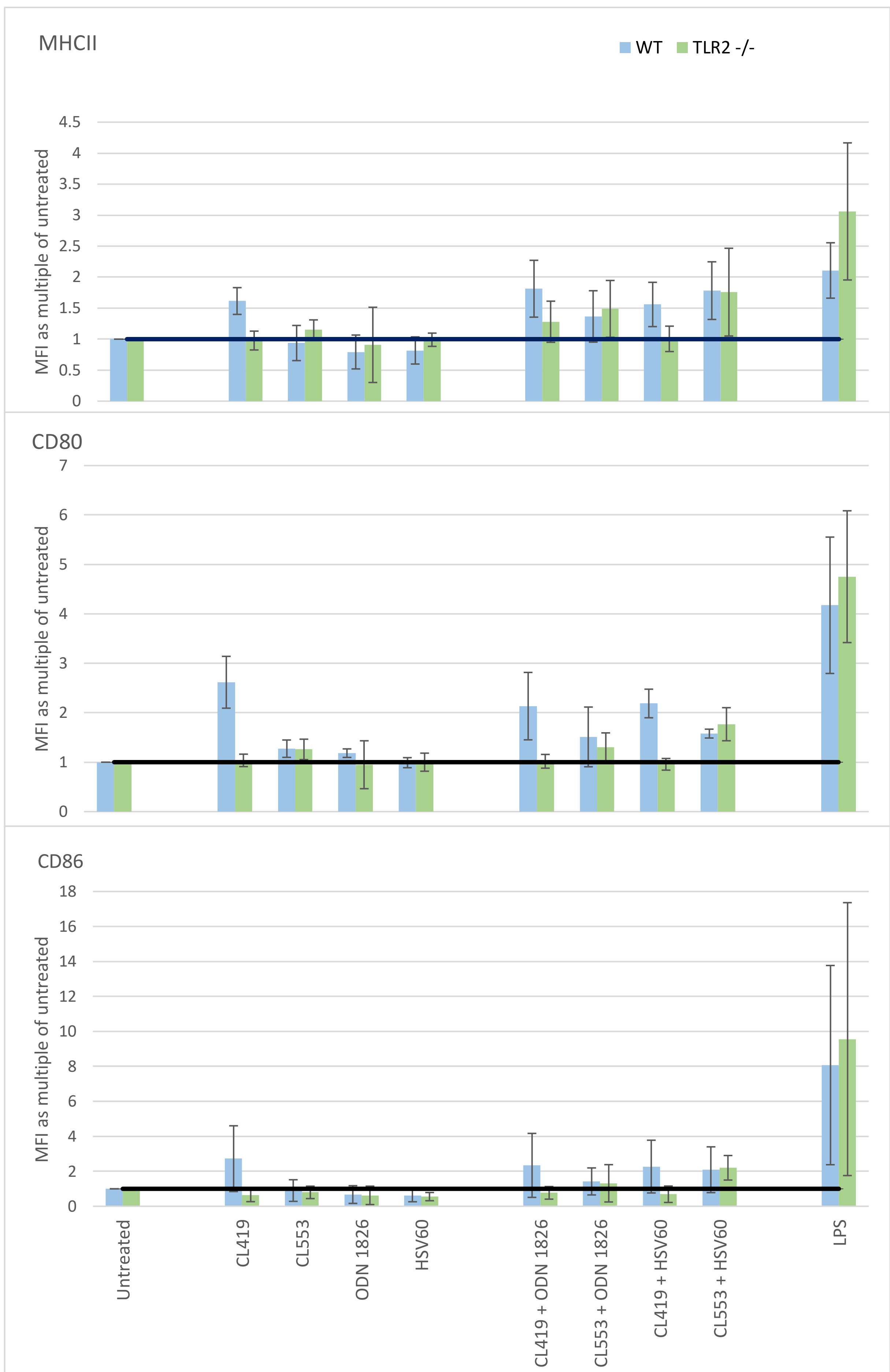
5.6.1. Trials including BMDC obtained from WT and TLR2-deficient mice

In order to investigate the TLR2-dependancy of the stimulation of BMDC by CL419/CL553 the same trials were repeated in BMDC obtained from TLR2-deficient mice (n=4) in direct comparison to WT BMDC (n=4) (3.10.) as depicted in figure 15.
In these trials unfortunately no significantly higher expression of CD86 could be observed except for the positive control (LPS). For these result the SD is considerably high, so the results may be due to high interindividual differences. Also, an error in processing could be the cause of this outcome.

In WT BMDC CL149 and CL553 could yield a significant upregulation of CD80 and MHCII. Also, ODN 1826 alone yielded a modest but significant upregulation of CD80.
The upregulation was higher when complexes of nucleic acids and liposomes were used compared to the use of either substance class (liposome, nucleic acid) alone. LPS yielded a similar upregulation for MHCII as the complexes, but a twofold to fourfold higher upregulation of CD80 and CD86.

In TLR2-deficient BMDC only CL553 could yield a modest upregulation of CD80 and CD86. Neither ODN 1826 nor HSV-60 alone could cause a significant increase of surface markers in this concentration, equal to the trial that was performed with WT cells.
ODN 1826 and HSV-60 could not yield a significant upregulation of surface markers when complexed with CL419. When complexed with CL553, HSV-60 yielded a modest but significant upregulation of all surface markers and ODN 1826 could yield a significantly higher expression of MHCII on BMDC. The LPS control showed a twofold to fivefold higher upregulation of surface markers compared to the best performing sample (HSV-60+CL553).

In overall terms the activation of BMDC by reagents including CL419 or CL553 was lower in TLR2-deficient cells compared to WT. Only CL553 complexed with ODN 1826 or HSV-60 could yield considerable activation (tab. 22-27).



MHCII
WT
TLR2 -/-
MFI as multiple of untreated
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CD80
7
6
5
4
3
2
1
0
MFI as multiple of untreated
CD86
18
16
14
12
10
8
6
4
2
0
MFI as multiple of untreated
Untreated
CL419
CL553
ODN 1826
HSV60
CL419 + ODN 1826
CL553 + ODN 1826
CL419 + HSV60
CL553 + HSV60
LPS

Fig. 15: Comparison of WT (light blue) and TLR2 deficient (light green) BMCD treated with the best performing nucleic acids (ODN 1826, HSV-60) complexed with CL419/CL553: upregulation of MHCII, CD80, CD 86

6. Discussion

Different trials were performed in order to investigate the effect of various nucleic acid-based the components complexed with liposomes to serve as an adjuvant of a potential nano-vaccine on DC required to induce immune responses as e.g. anti-tumor vaccination approaches. As stated in the introductory part of this doctoral thesis work, DC play an important role in anti-tumor immune therapy by initiating T cell responses. That is the reason why DC were in the focus of this research.

The fundamental structure for a nano-vaccine is a nano carrier that targets the relevant cells, in this case DC. The two liposomes that were chosen for trials (CL419 and CL553) show the ability to bind TLR2 (and TLR7) and activate APC via TLR triggering.

Different types of oligonucleotides were included in the trials, taking advantage of their capacity to be complexed by positively charged liposomes.

These oligonucleotides are aimed to act as additional adjuvants provoking an immune response via TLR9 (CpG oligonucleotides ODNs) or the STING (virus-derived oligonucleotides) pathway, yielding a possible synergistic effect in combination with the TLR2 activating capacity of the liposomes.

In the following the results of the trials that were performed will be concluded and discussed in the context of the current scientific knowledge.

6.1. Summary of the results

Both CL419 and CL553 were able to bind and in a dose-depending manner also to activate BMDC.

In order to demonstrate synergistic effects of the combined use of TLR9 and STING agonists, CL419 and CL553 were complexed with the respective substances in a concentration at which the liposomes showed a half-maximal activation of cells.

Different synthetical CpG-rich ODN, acting as TLR9 agonists, were tested in a soluble form and complexed with the well-established transfection reagents jetPEI and DOTAP. These single stranded ODNs manufactured by Eurofins Genomics that were chosen for the trials were ODN D-SL01, ODN BW006, ODN 2395, ODN M32, ODN D-SL03.

When complexed with jetPEI no significant effects of BMDC activation were observed. Thus, we continued using DOTAP as transfection agent. When complexed with DOTAP all of the three ODNs induced a higher activation of BMDC, which shows that DOTAP is a sufficient

transfection agent that was accordingly used for the following trials. DOTAP alone did not yield any upregulation of the assayed surface markers.

Three different virus-derived oligonucleotides (ISD, HSV-60, VACV-70) were tested in a soluble form and after complexation with the transfection reagent DOTAP. These double stranded oligonucleotides are ligands of the cytosolic DNA sensors (CDS) STING. When complexed with DOTAP, HSV-60 at a high concentration (1µg/ml) could yield a significant increase of CD80 and CD86 on the surface of BMDC. ISD and VAVC-70 unfortunately did not cause an increase of surface markers on BMDC. This may root in errors in storage or processing. To rule our errors in processing and storage the subsequent experiments were performed with a new stock of viral oligonucleotides.

TLR2-targeting liposomes were complexed with some of the evaluated ODNs (ODN D-SL-01, ODN D-SL03, ODN 1826) according to protocol. We observed the highest activation of BMDC by ODN after complexation with DOTAP. ODN1826 (complexed with DOTAP) yielded the highest activation. An activation of BMDC also occurred when treated with complexes of ODN1826 and CL419.

In a next step the liposomes were complexed with the virus-derived oligonucleotides (HSV-60, ISD, VACV-70) according to protocol. The highest DC activation could be observed after stimulation with HSV-60 in complexes with DOTAP. A modest activation was also observed after treatment with CL419 in complexes with HSV-60 and VACV-70.

In order to demonstrate TLR2-dependent activation of the liposomes CL419 and CL553 different trials were performed using WT and TLR2-deficient BMDC obtained from mice. The oligonucleotides ODN1826 and HSV-60 were used in the experiment considering the latest results.

The highest upregulation in WT BMDC was observed when CL419 was complexed with either one of the oligonucleotides. The activation of WT BMDC was higher when complexes of nucleic acids and liposomes were used compared to the use of either substance class (liposome, nucleic acid) alone. These results suggest a synergistic effect on BMDC activation when two different substance classes are used that induce different pathways of BMDC activation.

In TLR2-deficient BMDC, ODN 1826 and HSV-60 could not yield a significant upregulation of surface markers when complexed with CL419. When complexed with CL553 HSV-60 yielded a modest but significant upregulation of all surface markers and ODN 1826 could yield a significantly higher expression of MHCII on BMDC. Also, CL553 alone, but not CL419, yielded a modest upregulation of surface markers. As stated previously, CL553 is also a TLR7 ligand and therefore the observed effect on BMDC activation can be attributed to the TLR7

targeting of CL553. Since the effect is still lower compared to the effect of the complexes WT BMDC these results are still consistent with the theory that CL553 activates BMDC via TLR2. For any probes including CL419 there was no significant increase of the expression of surface activation markers observed, so this way it is proven that the activation of BMDC by CL419 takes place via TLR2.

6.2. Implications of the results

After dose titration of the TLR2-targeting liposomes and the different types of nucleic acids that were introduced before, the most potent nucleic acids in terms of DC stimulation were selected to form complexes with CL419 and CL553. The aim was to use both substance classes at a lower concentration inducing different pathways in DC that yield a synergistic DC stimulation. Accordingly, the liposomes were used in a concentration where a half-maximal effect had been observed.

As the results show in 5.5., nucleic acids complexed with CL419 or CL553 were able to induce a higher expression of surface markers compared to the nucleic acids or CL419/CL553 alone. It can be concluded that this effect is the result of simultaneous stimulation of DC by different pathways. The induction of the different pathways in DC is followed by the production of different kinds of interleukins (see 2.5.1.1. and 2.5.1.2.), inducing different stimuli for T cells. The consequences of these results may be, that in a future nano-vaccine generated from liposome/nucleic acid complexes, including a tumor-specific antigen, the concentration of liposomes and nucleic acids can be held lower than when used alone, preventing toxic effects and lowering the costs.

It is possible that a combination of substances acting synergistically can cause higher effects compared to one substance alone. So, this way the effectiveness of cancer therapies could be improved.

The use of a tumor-specific vaccine may also facilitate the reduction of the dosage of less specific therapies like chemotherapy and radiation, thereby also reducing their adverse events. In human anti-tumor therapy interindividual differences may be leveled out by using different substance classes. If tumor cells of one individual do not react as strongly as others to the treatment with one of the substances, as we had been able to observe in some trial, this bad response could be compensated by the use of another substance. So, the use of two substances initiating an immune reaction lowers the risk of a poor response to the tumor treatment.

6.4. Limitations of the interpretation

Even though this work could prove that the tested substances can provoke a DC activation in-vitro, it is not verified that this nano vaccine also serves as an effective tumor therapy in a living organism. From in vitro assays no conclusions can be drawn in regard to the in vivo pharmacodynamics and pharmacokinetics or the toxicity of the vaccine. Furthermore, the effect on tumor-bearing individuals cannot yet be determined. It is to be investigated whether the microenvironment of the tumor prevents the success of the therapy, even when the immune response of the individual is sufficient. The reaction of the tumor mass and the overall survival of the individual needs to be investigated in order to determine the applicability of the vaccine to treat melanoma.

Altogether, tumor therapy is a complex matter. There will not be a universal treatment to cure every tumor. A concept integrating different anti-tumor therapies might be a promising approach. Only activating the immune system might be not effective for fighting cancer, due to its limitations. Neither is chemotherapy, radiation or checkpoint therapy a satisfactory concept to eradicate the different kinds of cancer. The simultaneous application of complementary therapies could result in better outcomes by covering the deficiencies of each therapy alone.

6.3. Reflection of the results in the context of the current scientific knowledge

DC-addressing anti-tumor vaccines are a promising approach in anti-tumor therapy due to the characteristics and functions of DC. DC orchestrate essential portions of the adaptive immune response to antigens, they are able to migrate from lymphoid to non-lymphoid tissues and regulate essential cytokine release. One more important characteristic, predestining DC as targets for immunotherapeutic approaches, is their natural ability to uptake and process pathogenic particles via distinct receptors. Modified nano-carriers, aimed to bind those specific DC receptors, facilitate the uptake of the nano-carrier by DC and the delivery of the incorporated antigen [48].

The characteristics of nano-carriers influence the development and immune response of DC. Different types of nano-carriers, addressing DC, have been investigated.

Liposomal nano-carriers showed low toxicity in vivo [58]. Toxic effects on liver, lungs or other organs of the TLR2 binding liposomes that we used in our study, remain to be examined.
The utilization of liposomal nano-carriers revealed different possibilities, for one the variation of the composition of lipids resulting in different particle size, charge etc. Secondly adjuvants, addressing specific DC receptors can be conjugated to liposomes. Different antigens can be incorporated by liposomes [55].
In our study we showed, that the liposomes CL419 and CL553 can address DC directly via TLR2 (and TLR7) receptor. Not only were we able to address DC, but we also observed a DC activation after stimulation with the TLR2 binding liposomes, functioning themselves as adjuvants. These liposomes are positively charged and therefore bear the ability to bind negatively charged cargo, which we took advantage of to generate liposome/oligonucleotide complexes.
Oligonucleotides derived from pathogens, targeting APC via PRR (e.g. TLR, STING), have successfully been utilized as adjuvants in liposomal nano-carriers, yielding a better immune response compared to the oligonucleotide as an adjuvant in a soluble form [58]. We included virus-derived oligonucleotide and CpG rich ODN for complexation with the liposomal carriers, after evaluating the oligonucleotides effects alone on DC in vitro. We chose those oligonucleotides that already provoked a stimulation of DC in a soluble form.
It has been reported, that cationic nano-carriers conjugated with an adjuvant and antigen showed a better immune response compared to the adjuvant and antigen without carrier [55]. We were able to make similar observations. Oligonucleotides, acting as adjuvants, yielded a higher DC activation in vitro, when complexed with one of the TLR2 binding liposomes than in a soluble form.
TLR targeting adjuvants have been studied in mice, yielding an effective immune response and DC activation. However, substantial studies examining TLR dependent activation of DC in humans have not been completed [57]. Concludingly, it is unsure to say, whether the effects we observed in murine BMDS will also be translatable to humans.
Furthermore, the application of DC targeting anti-tumor vaccines needs to be discussed considering the advantages and disadvantages of a ex vivo and in vivo vaccination of DC. One approach is to directly apply the DC targeting vaccine into the patients' system. Different routes are possibly for this in vivo application e.g. oral/mucosa uptake, intravenous or local application. Another possibility is the ex vivo treatment of precursor DC, isolated form the patient. The isolated DC are stimulated in the laboratory and then reapplied to the patients'

system. It is assumed, that the maturation of DC differs as between ex vivo and in vivo stimulation [56].
Ex vivo manipulated DC vaccines have shown low effects in tumor therapy, which may be attributed to the lack of ability of ex vivo manipulated DC to overcome the tumor microenvironment (TME) [78].
Studies suggest, that in vivo vaccination of DC may be the more potent approach for anti-tumor therapy, mimicking the more natural way of DC activation[56].
In order to defeat the TME a complex immune response is necessary. DC may yield a major component of anti-tumor immune response, yet it is proposed that other cells like Th1 cells and natural killer cells play an important role in anti-tumor immunity [80].
In order to efficiently fight immunosuppressive tumors those cell types need to be further investigated and addressed by a potential therapy. It is to be critically examined whether a sufficient anti-tumor therapy can be attained by DC vaccination alone, or if additional strategies are requires.
The combination of conventional tumor therapies e.g. chemotherapy/radiation and DC vaccination might offer new possibilities and better therapy results. Especially considering the recent discovery, that chemotherapy and radiation harm immunosuppressive cells in the TME and thereby support the tumor immunity. It is suggested, that the two therapy strategies combined might work mutually supportive [79].
In ideal anti-tumor therapy strategy would include a personalized combination of therapies, comprised of DC vaccination and a conventional tumor therapy, dependent on the tumors' microenvironment and characteristics of the tumor (e.g. the immunosuppressive molecules of the tumor surface).

6.5. Outlook

In these trials in order to measure DC activation, only the surface markers were used. The surface markers, that were chosen are well suited to estimate the activation of BMDC, because they are expressed proportionally to the activation of DC. Nevertheless, trials measuring the quantity of interleukins should be performed, in order to measure the "communication" between DC and T-cells.
Also, the T-cell activation can be measured itself. Therefore, after performing trials for DC activation, the stimulated DC can be co-cultured with T-cells (Th1 and Th2). The level of T cell activation can be analyzed in the fluorescence cytometer, using T cell specific surface markers, analogous to the DC analyses.

Further, in vivo trials in healthy (non-cancerous) mice need to be performed in order to examine the pharmacokinetics and pharmacodynamics as well as the tolerance of the vaccine. In a dose finding study the maximal tolerated dose which also shows a significant effect on the immune response needs to be detected.

In this step, the application form of the vaccine has to be taken into consideration. If the vaccine is administered orally, there is the risk of an inactivation by the gastric acid and a high first pass effect in the liver. The CYP enzymes located in the liver, responsible for this effect, can cause a metabolization of the vaccine which may result in an inactivation of the substances. Consequently, for oral applications the bioavailability is poorly controllable. When partly inactivated by the liver, dose adjustments (i.e. increases) need to be made, increasing the costs and the toxicity.

An option avoiding the first pass effect, thus increasing the bioavailability, would be an intravenous (i.v.) application of the vaccine. Consequently, the applied dosage could be maintained at a lower level. Nevertheless, the substances used in the vaccine could accumulate in other organs like the lungs or the spleen. Also, an i.v. application of the vaccine leads to different disadvantages. For intravenous applied medication, an i.v. access is required. In consequence, the medication cannot be taken by the patient at home, but only administered by a physician (or other medical staff). If the administration of the vaccine needs to be on a regular basis, this could cause organizational difficulties. Another problem of an i.v. application is the risk of infection. Every i.v. access poses the risk of a local (i.e. abscess, lymphangitis) or systemic (i.e. sepsis) infection.

Another option could be a transcutaneous or intracutaneous application, considering that immature DC are present in different kind of tissues, including the skin. Lipophilic substances are non-polar allowing them to pass the lipid bilayer of the skin. CL419 and CL553 bear lipophilic properties and are accordingly suited for a transcutaneous application.

Additional experiments need to be performed with melanoma bearing mice, observing the effects of the vaccine on the tumor mass (tumor growth, metastasis) and the organism (all over survival rate, live quality). In those tumor bearing mice trials including an intratumoral application of the vaccine could be performed, directing the substances to where exactly they are needed within the body. This seems to be an option for melanoma, which is a tumor of the skin in therefore easily traceable and accessible. Unfortunately, this application form might not be so easily applicable for other types of tumors, but well suited in our case.

In a second dose finding study the maximal tolerated dose which also shows a significant effect on the immune response needs to be detected.

This will be an important step in the direction of developing a sufficient cancer therapy.

In order to obtain the pharmaceutical approval in humans a IV phase clinical study needs to be performed with human test subjects. Phase I includes the testing of a small group of healthy volunteers, in order to ensure the safety of the treatment and to examine the distribution within the body. In phase II, a small group of volunteers bearing the disease is tested for the right dosage and the effectiveness of the treatment. Normally, there is a control group involved. Phase III involves a larger group of patients, examining a wide variety of people with respect to adverse events and effectiveness. There can be different treatment groups and always a control group. After approval, phase IV can be initiated, implying the common use of the medication on the market.

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Appendix

1. SDS Page

The same amount of pDNA has been incubated with increasing amounts of CL419 for 20 min. As shown above CL419 does not move when not complexed. pDNA itself moves up to a certain point. In column 1 (1µg CL419) a certain amout of the pDNA has been complexed with CL419, as there appears a second line apart from the pDNA mark. From column 2 (2µg CL219) and higher concentrations of CL419 all of the pDNA is in complex with CL419, as the line of pDNA disappears completely and the second line increases its intensity.

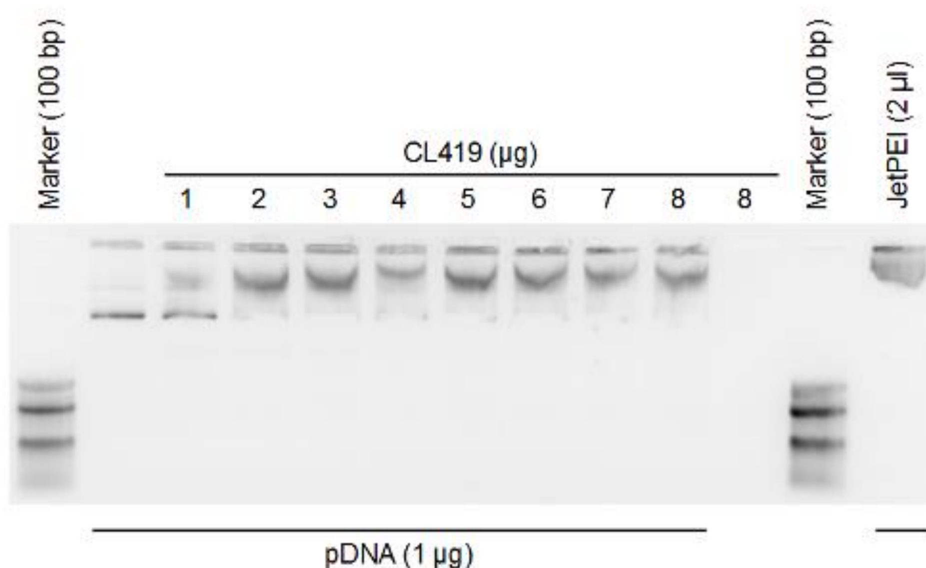
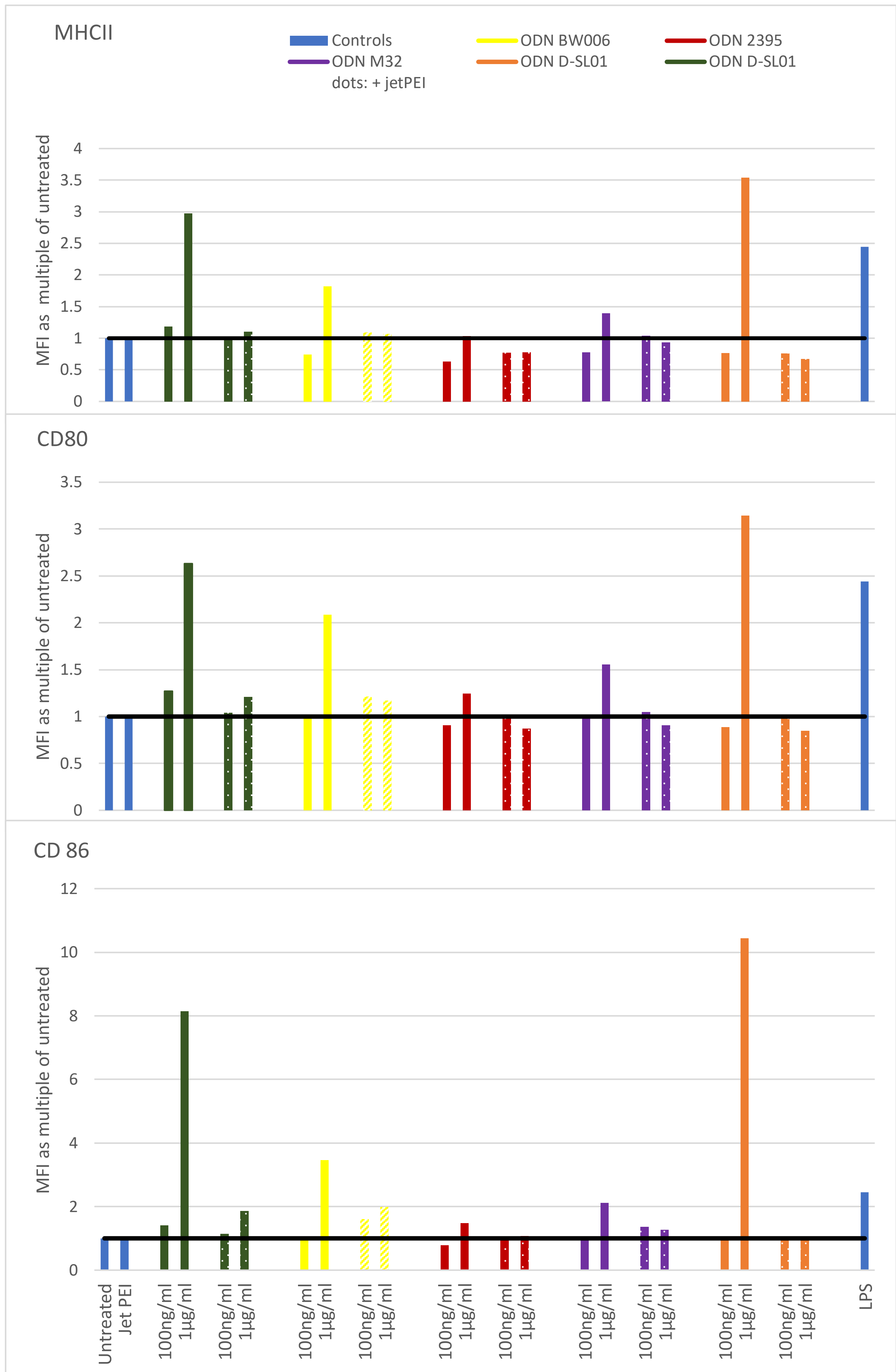


Fig. Appendix 1: SDS Page of Complexed formed by CL419 and pDNA plus controls (only pDNA, only CL419, markers and internal control (JetPEI)) performed by Matthias Bros.

2. Additional figures from the results

2.1. ODN's complexed with jetPEI

Fig. Appendix 2 (see next page): BMCD treated with ODN D-SL01 (dark green), ODN D-SL03 (orange), ODN BW006 (yellow), ODN 2395 (dark red), ODN M32 (purple) in a soluble form and complexed with jetPEI (dotted bars): upregulation of MHCII, CD80, CD86.


MHCII
Controls
ODN BW006
ODN 2395
ODN M32
ODN D-SL01
ODN D-SL01
dots: + jetPEI
MFI as multiple of untreated
4
3.5
3
2.5
2
1.5
1
0.5
0
CD80
MFI as multiple of untreated
3.5
3
2.5
2
1.5
1
0.5
0
CD 86
MFI as multiple of untreated
12
10
8
6
4
2
0
Untreated
Jet PEI
100ng/ml
1µg/ml
LPS

2.2. Viral oligonucleotides in complexes with CL419/CL553 and DOTAP

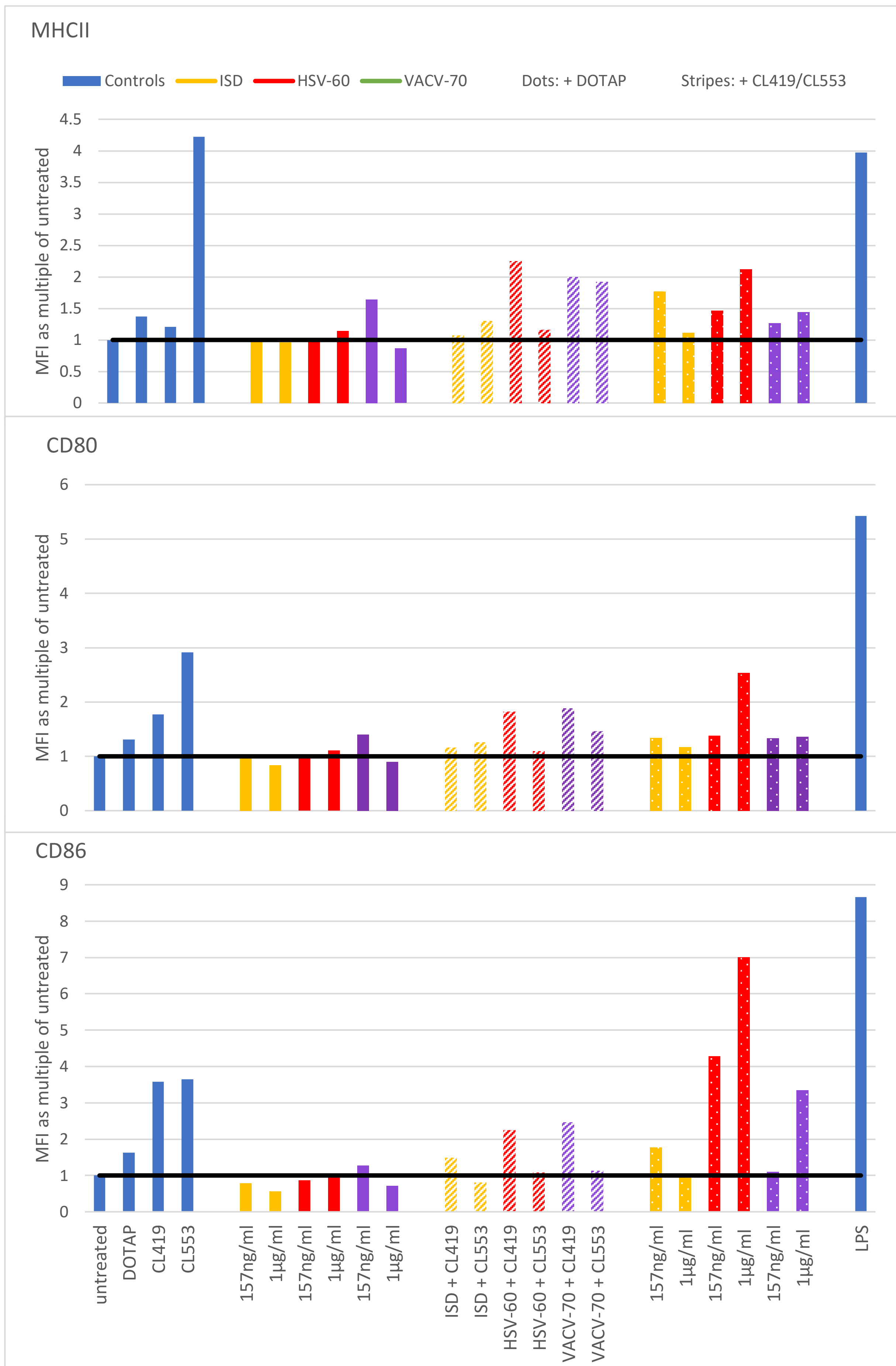


Fig. Appendix 3: BMCD treated with viral oligonucleotides (ISD (dark yellow), HSV-60 (red), VACV-70 (violet)) in a soluble form and complexed with DOTAP (dotted bars) or CL419/CL553 (striped bars): upregulation of MHCII, CD80, CD86.

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