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**Effects of commonly used antiviral
vaccines on human plasmacytoid
dendritic cells**

Dissertation

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List of abbreviations

7-AAD	7-amino-actinomycin D
ACP	Ammonium chloride potassium
AIM-V	Adoptive Immunotherapy Media
APC	Allophycocyanin
BDCA	Blood dendritic cell antigen
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluoresceindiacetate succinimidyl ester
CpG	Cytosine-phosphate-guanosine motif
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
GrB	Granzyme B
HLA	Human leucocyte antigen
HRP	Horseradish peroxidase
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPV	Inactivated polio vaccine
IRF7	Interferon regulatory factor 7
Lin-1	Lineage 1, cocktail containing antibodies against CD3, CD14, CD16, CD19, CD20, CD56
MAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
Min	Minute
MLR	Mixed lymphocyte reaction
MOI	Multiplicity of infection

MV	Measles vaccine
NF- κ B	Nuclear factor kappa B
NK cell	Natural killer cell
ODN 2006	Oligodesoxynucleotide class B, sequence: 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' on a phosphorothioate backbone
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline (without calcium and magnesium)
PDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PECy	Phycoerythrin tandem with cyanin dye
PerCPCy	Peridinin chlorophyll protein tandem with cyanin dye
PI	Propidium iodide
RPMI	Roswell Park Memorial Institute, common cell culture medium
RT	Room temperature
RV	Rubella vaccine
ssRNA	Single stranded ribonucleic acid
TBEV	Tick-borne encephalitis vaccine
TcR	T cell receptor
TLR	Toll-like receptor
Treg	Regulatory T cell
YFV	Yellow fever vaccine

1. Introduction

1.1 Plasmacytoid dendritic cells between immunogenic and tolerogenic properties

Plasmacytoid dendritic cells (pDCs) are a rare and special type of immune cell that comprise between 0.2 and 0.8% of all peripheral blood mononuclear cells (PBMCs) [164]. They were first described as T-associated plasma cells in 1958 by Lennert and Remmele as they were discovered in T cell areas of reactive lymph nodes and expressed a plasma cell like morphology [105]. Several different denominations such as plasmacytoid T cells [46], plasmacytoid monocytes [44] or pDC precursors [62] have subsequently been attributed to these cells that are today known as pDCs, demonstrating the former nescience about the function and origin of pDCs. It was not until 1999 that pDCs were finally identified as natural interferon producing cells [23, 152], a cell type capable of producing large amounts of type I interferons (IFNs) upon viral infections [47]. After the characterisation of human pDCs, these cells have also been discovered in mice [12, 124]. Human and murine pDCs display many similarities concerning both surface molecule expression and functional properties [75, 140].

Currently, the origin of pDCs is still controversial. PDCs express a variety of genes characteristic for the lymphoid lineage [141]. Additionally, signalling pathways very similar to that of B cells have been revealed in pDCs [21] indicating a lymphoid origin of pDCs. However, other findings from mouse studies including the identification of different pDC subsets in murine bone marrow [128] or the fact that myeloid progenitor cells could also develop into pDCs and still express lymphoid genetic markers [151] oppose that view. Some studies on murine pDCs argue for the existence of a common dendritic cell precursor giving rise to conventional and plasmacytoid dendritic cells [30, 123]. A recent model from Reizis attempts to take all these diverging findings into account and suggests that pDCs might be a heterogenous population with the majority of pDCs arising from a common dendritic precursor cell and with the lymphoid lineage contributing to some extent to pDC development [139].

The surface phenotype of human pDCs can be characterised as cluster of differentiation (CD)4⁺, CD45RA⁺, human leukocyte antigen (HLA)-DR⁺, CD123^{high},

blood dendritic cell antigen (BDCA)-2⁺, BDCA-4⁺, CD11c⁻ and lineage⁻ [39, 41, 164]. Lineage⁻ means that pDCs lack the expression of commonly known antigens associated with PBMCs like B cells (CD19, CD20), T cells (CD3), monocytes (CD14) or natural killer (NK) cells (CD16, CD56). BDCA-2 and BDCA-4 are uniquely detected on the surface of pDCs in human peripheral blood [41]. BDCA-2 belongs to a group of surface antigens called C-type lectins and is involved in antigen capturing and presentation. Engagement of BDCA-2 downregulates IFN production by pDCs [40]. BDCA-4 is identical to neuropilin-1, a neuronal receptor for axon guidance factors, that also functions as receptor for vascular endothelial growth factor-A expressed by endothelial cells and tumour cells [41, 70, 154]. Due to the univocal expression of BDCA-2 and BDCA-4 by pDCs among human peripheral blood cells, these two surface proteins can be used to identify pDCs via flow cytometry.

PDCs are mainly located in secondary lymphoid tissues such as peripheral lymph nodes which they reach and enter hematogenously through high endothelial venules [23, 35, 177] though they can also be found in organs e.g. the lung or kidneys [113, 175]. Isolated pDCs die rapidly under cell culture conditions without supplementation of certain prosurvival stimuli like interleukin (IL)-3 or CD40 ligand [62].

Nowadays, pDCs claim their role as important mediators in antiviral immunity. In humans, they selectively express the toll-like receptors (TLR) 7 and 9 that act as sensors for viral infections [87, 90]. TLRs represent a large subset of pattern recognition receptors expressed by various immune cells and recognise conserved microbial antigens, so called pathogen-associated molecular patterns (PAMPs) [57]. In case of the pDC TLRs 7 and 9, single-stranded ribonucleic acid (ssRNA) [36, 71, 112] or deoxyribonucleic acid (DNA) with a 2' deoxyribose phosphate backbone serve as PAMPs, respectively [64, 72]. These ligands of TLR 7 and 9 are frequently encountered in viruses, and in fact many studies have been published demonstrating the recognition of various viruses including herpes simplex virus type 1 and 2 [99, 111], murine cytomegalovirus [100], influenza virus [112], hepatitis C virus [163] etc. by both human and murine pDCs via TLRs. While most immune cells express TLRs on the cell surface, TLR 7 and 9 are located in the endosomal compartment in pDCs, thus protecting the immune system against recognition of self-DNA [14]. The stimulation of TLRs initiates a signalling cascade leading to the activation of transcription factors such as nuclear factor kappa B

(NF- κ B) and interferon regulatory factor 7 (IRF7). NF- κ B in turn promotes transcription of proinflammatory cytokines like IL-6 or tumour necrosis factor alpha [14, 91]. However, activation of IRF7 is even more important in pDCs. IRF7 is constitutively highly expressed by pDCs [84] and is considered as “master regulator” of the interferon response upon viral infections [78]. The high expression levels of IRF7 enable pDCs to produce large amounts of type-I interferons in response to different viruses. Interestingly, pDCs begin to produce various subtypes of type-I interferons like IFN- α , IFN- β , IFN- ω or IFN- τ very rapidly. Interferon production upon stimulation with TLR agonists occupies about 50 % of total pDC transcription and reaches its peak after already 12 hours [82].

Besides the synthesis of proinflammatory cytokines, pDCs gain a mature phenotype with a more dendritic cell-like morphology after activation of TLRs or stimulation with prosurvival cytokines like IL-3, and subsequently upregulate costimulatory surface molecules like CD80, CD86 or HLA-DR [62, 63, 89]. This maturation process allows pDCs to interact with various cell types of the immune system.

Due to these properties – selective expression of TLR 7 and 9, production of high quantities of interferons and expression of costimulatory molecules after TLR-engagement – pDCs play a pivotal role in the course of viral infections and link innate and adaptive immune responses. On the one hand, type-I interferons secreted by pDCs lead to the transcription of multiple genes that exert antiviral functions and thus protect cells from further viral replication [158]. Furthermore, type-I interferons as well as direct interactions with pDCs can activate NK cells and thereby strongly support innate immune responses [6, 53]. On the other hand, a multitude of functions contributing to adaptive immunity have been described for pDCs in conjunction with type-I interferons. Consequently, pDCs can stimulate naive CD4⁺ T cell proliferation [89], promote either Th1 [24] or Th2 polarisation [81], induce and expand virus-specific CD8⁺ cytotoxic T cells [49, 146] and enhance migration of T cells [116]. Additionally, pDCs support the differentiation of B cells into plasma cells and the synthesis of immunoglobulins (Igs) [88, 134]. Type I interferons are also implicated in maturation and differentiation processes of conventional dendritic cells (DCs) [110, 145]. PDCs can act as antigen-presenting cells [172] and acquire the ability to crosspresent antigens after maturation [34, 77]. These examples illustrate the pleiotropic effects exerted by pDCs and type-I

interferons that trigger both innate and adaptive immune responses and underpin the prominent role of pDCs especially in the context of viral infections.

In addition to their crucial role as antiviral effectors, pDCs have been associated with a variety of diseases. In particular autoimmune diseases such as psoriasis or systemic lupus erythematosus seem to be at least partially driven by pDCs and high levels of type I-interferons [13, 115, 125]. In these cases, recognition of self-DNA activates pDCs and stimulates production of inflammatory cytokines [104, 115]. pDCs have also been detected in different cancers in humans and mice where their immunogenic properties appear to be altered and where they might contribute to the suppression of an appropriate antitumour response [69, 121, 171, 173].

In contrast to the indisputable immunogenic properties reported above, tolerogenic implications of pDCs have also been described. Some studies demonstrated that pDCs are capable of anergising T cells [102, 103]. Further, pDCs can induce CD4⁺ [83, 120] or CD8⁺ regulatory T cells (Tregs) [56] producing IL-10, a cytokine with anti-inflammatory characteristics [143]. Tregs are special immune cells which exert immunosuppressive effects and mediate self-tolerance [144]. Clinical tolerogenic examples reported for pDCs in mouse models are development of oral tolerance [59], suppression of asthmatic reactions [31], mediation of tolerance to allografts in transplantation [126] and inhibition of graft-versus-host disease [65]. One important pathway towards the induction of Tregs by pDCs seems to be the expression of the enzyme indoleamine 2,3-dioxygenase (IDO) [26]. The degradation of tryptophan and the production of kynurenines by IDO are thought to trigger immune suppression [45]. Recently, our group revealed another potential mechanism of pDCs towards immune suppression: IL-3-stimulated pDCs secrete large amounts of granzyme B (GrB) and potently suppress proliferation of allogeneic T cells in a GrB-dependent manner [86]. Apart from this finding, the role of pDC-derived GrB remains elusive and further studies have to reveal the function and regulation of GrB in pDCs.

1.2 Granzyme B – more than just a killer protease

Granzyme B is one of five known human granzymes (GrA, B, H, K, M). It is a serine protease that cleaves substrates after aspartic acid residues. Killing of virus-infected cells or tumour cells by cytotoxic T lymphocytes (CTLs) or NK cells

represents the classical function of GrB [3, 27]. In this context, GrB is stored in secretory lysosomes, also called lytic granules, together with perforin [106]. The lytic granules fuse with the plasma membrane of NK cells or CTLs upon recognition of target cells and thereby release their content into the extracellular space [27]. To induce cell death of infected or transformed cells, GrB has to enter the cytoplasm of target cells. This process is not entirely understood so far but it seems that perforin triggers endocytosis of GrB and the subsequent escape of GrB from endosomes into the cytoplasm [92, 165, 166]. Once within the target cell, GrB initiates apoptosis, a tightly regulated form of cell death which is carried out by a family of proteases called caspases [42]. Caspases degrade various antiapoptotic cellular proteins and activate proapoptotic substrates, finally leading to cellular demise. One way how GrB induces cell death is via direct activation of caspases [1, 5, 117, 176]. However, GrB is also able to directly cleave caspase substrates e.g. Bid [9, 160] or inhibitor of caspase activated DNAase [167] and thus induces the apoptotic cascade.

Apart from CTLs and NK cells, expression of GrB has been demonstrated for many other blood cell types like B cells [66, 67, 85], mast cells [159], basophils [170], macrophages [93] or hematopoietic progenitor cells [11]. GrB was first detected in human pDCs in 2002 [141]. PDCs are able to secrete abundant amounts of GrB upon stimulation with IL-3 and IL-10 but do not express perforin [86]. GrB expression has also been revealed in other cell types such as chondrocytes [79], keratinocytes [73], spermatogenic cells or syncytial trophoblasts of placental tissue [74] indicating alternative roles for GrB.

In fact, increasing evidence suggests several non-cytotoxic functions of GrB [20, 142]. For example, granzymes can directly cleave viral proteins and thus block further viral spreading independently of the induction of cell death [4, 94]. Extracellular granzyme B is implicated in receptor cleavage [51, 52] as well as matrix degradation and remodelling [19, 28, 50]. Cleavage of extracellular proteins affects tumour cell invasion and might also influence lymphocyte migration [19, 20]. Further, GrB seems to exert immunosuppressive effects. GrB produced by pDCs or Tregs is involved in the inhibition of T cell proliferation in a perforin-independent manner [58, 86]. Studies in mice associated GrB with the activation-induced cell death of T cells, a process contributing to control the number of activated lymphocytes [33, 150].

In a clinical context, elevated levels of GrB have been detected in bronchoalveolar lavages of patients suffering from asthma [16, 170] or chronic obstructive pulmonary disease [76] as well as in sera and synovial fluids of patients with rheumatoid arthritis [155, 162]. However, future studies have to answer the questions whether these elevated levels of GrB play a pathogenetic role and whether GrB release is just a consequence of the accumulation and activation of lymphocytes at the site of inflammation or a regulated process [20]. Especially, the involvement of pDCs as abundant source of secreted GrB deserves further evaluation.

1.3 Hypothesis and aims

As the discovery of the ability of pDCs to secrete copious amounts of GrB has been made quite recently [86], knowledge about pDC-derived GrB is still scarce. Thus, we wanted to further investigate the regulation and function of GrB in pDCs in the present study.

Due to the fact that pDCs are crucial mediators in innate and adaptive antiviral immune responses, we hypothesised that commonly used antiviral vaccines have the ability to modulate pDCs and hence also pDC-derived GrB. Such prophylactic vaccines generally contain either whole virus particles in an attenuated or inactivated form or just parts of virus particles e.g. viral proteins [137]. Consequently, vaccines might contain TLR agonists like ssRNA or DNA and therefore might be effectively recognised by pDCs. Indeed, a recent study has proven the capacity of vaccines to activate different subsets of TLRs [149].

The precise mechanisms how these vaccines confer long-lasting immunity after vaccination are not fully understood yet. While effects of vaccines on B cells and antibody production are well described [2, 131], the influence on other immune cells, including dendritic cells or T cells, are rather poorly understood.

Thus, the goal of this study was to investigate effects of antiviral vaccines on pDCs with special regard to the regulation of GrB in order to shed more light into the overall immune response after vaccination.

We hypothesised that prophylactic vaccines have the ability to activate pDCs, leading to an immunogenic phenotype. In order to assess the activation of pDCs upon stimulation with antiviral vaccines, we intended to examine the upregulation of costimulatory molecules and the secretion of IFN- α by pDCs. For that purpose,

several commonly used vaccines against polio, yellow fever, measles, tick-borne encephalitis and rubella were evaluated.

Based on the recent findings that GrB-secreting pDCs potently inhibit allogenic T cell proliferation in a GrB-dependent manner [86], we further postulated that a vaccine-induced immunogenic pDC phenotype is accompanied by reduced intracellular and secreted amounts of GrB, resulting in an increased potential of pDCs to induce T cell expansion.

2. Materials and methods

2.1 Isolation of distinct cellular subtypes from fresh blood or buffy coats

For isolation of plasmacytoid dendritic cells and T cells, buffy coats were obtained from the German Red Cross in Ulm. Additionally, 50 ml of fresh blood were collected from healthy individuals for isolation of T cells used for mixed lymphocyte reactions (MLRs).

2.1.1 PBMC purification

In order to isolate different cellular subtypes from fresh blood and buffy coats, PBMCs had to be purified. Therefore, fresh blood or buffy coats were diluted 1:2 with phosphate buffered saline (PBS, Biochrom AG, Berlin, Germany) and 35 ml were layered on 15 ml of Biocoll Separating Solution (Biochrom AG, Berlin, Germany) in 50 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for density gradient centrifugation (1000 x g, 20°C, 15 minutes (min), brakes off). The white rings were harvested into new 50 ml tubes and washed with PBS (300 x g, 20°C, 10 min). Pellets were resuspended, pooled in one tube and washed with PBS again (200 x g, 20°C, 15 min). Next, PBMCs were incubated with 10 ml of ammonium chloride potassium (ACP) lysing buffer for 7 min at room temperature (RT) to lyse remaining erythrocytes and then washed again with PBS (300 x g, 20°C, 10 min). For ACP lysing buffer, distilled water (AlleMan Pharma GmbH, Rimbach, Germany), 0.15 M ammonium chloride (Merck KGaA, Darmstadt, Germany), 10 mM potassium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M ethylenediaminetetraacetic acid (EDTA, Carl Roth, Karlsruhe, Germany) were admixed, filtered sterile and adjusted to a pH between 7.2 and 7.4. Pellets were resuspended in 50 ml of PBS and PBMCs counted in a Neubauer counting chamber (Laboroptik, Friedrichsdorf, Germany). In order to proceed to magnetic isolation of distinct cellular subtypes, PBMCs were spun down (300 x g, 20°C, 10 min) and washed once (300 x g, 20°C, 10 min) with magnetic-activated cell sorting (MACS) buffer. MACS buffer was made of PBS, 0.5% bovine serum albumin (BSA, Serva Electrophoresis GmbH, Heidelberg, Germany) and 2 mM

EDTA, filtered sterile, degassed and adjusted to a pH between 7.2 and 7.4. Supernatants were discarded and pellets contained purified PBMCs.

2.1.2 PDC positive selection

For purification of pDCs for all experiments except MLRs and spinning disc confocal microscopy, the CD304 (BDCA4/neuropilin-1) Microbead Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used according to the slightly modified manufacturer's protocol. Pellets containing the PBMCs were resuspended in 150 µl of MACS buffer for 10^8 cells. 50 µl of FcR (Fc receptor) Blocking Reagent and 50 µl of CD304 (BDCA4/neuropilin-1) MicroBeads per 10^8 cells were added, followed by an incubation period of 15 min at 4°C in the dark. After a washing step with MACS buffer (300 x g, 20°C, 10 min), supernatants were discarded and cells were resuspended in 500 µl of MACS buffer per 10^8 cells in order to proceed to MACS separation. Therefore, LS columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were placed in a MACS separator (quadroMACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and rinsed with 3 ml of MACS buffer. Cell suspension was applied onto the columns. Columns were washed three times using 3 ml of MACS buffer. Flow-through contained unlabelled PBMCs whereas labelled (hence positively selected) pDCs were retained in the column. PDCs were flushed out with 5 ml of MACS buffer by firmly applying a plunger. In order to increase purity of pDCs, the eluted fraction was again added to a new LS column. Finally, pDCs were counted, resuspended in medium and seeded. Purity of pDCs was predominantly higher than 90%.

2.1.3 PDC negative selection

For mixed lymphocyte reaction experiments and spinning disc confocal microscopy, unlabelled pDCs were used and isolated with the aid of the Plasmacytoid dendritic cell isolation kit human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the slightly modified manufacturer's protocol. PBMCs were resuspended in 200 µl of MACS buffer per 10^8 cells. After adding 50 µl of PDC Biotin-Antibody Cocktail (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) per 10^8 cells containing a mixture of monoclonal biotin-conjugated antibodies against antigens that are not expressed by human pDCs, cells were

incubated for 10 min at 4°C in the dark. The labelled cells were then washed twice with MACS buffer (300 x g, 20°C, 10 min). Supernatants were discarded and pellets were resuspended in 200 µl of MACS buffer per 10⁸ cells. 50 µl of Anti-Biotin MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added per 10⁸ cells, followed by another incubation period (4°C, 15 min, dark). The cell suspension was washed again (300 x g, 20°C, 10 min), supernatants were discarded and 10⁸ PBMCs were resuspended in 500 µl of MACS buffer. LD Columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were placed in a MACS separator and rinsed with 2 ml of MACS buffer, 500 µl of cell suspension were applied per column and columns were washed twice with 1 ml of MACS buffer. The flow-through containing the unlabelled (hence negatively selected) pDCs was collected, cells were counted, resuspended in medium and seeded. Purity of pDCs was regularly higher than 95%.

2.1.4 CD4⁺ T-cell isolation

CD4⁺ T cells were isolated by using the CD4⁺ T Cell Isolation Kit II human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the slightly modified manufacturers' protocol. 10⁷ PBMCs were resuspended in 40 µl of MACS buffer and 10 µl of CD4⁺ T Cell Biotin-Antibody Cocktail (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) containing biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, T cell receptor (TcR)γ/δ and glycophorin A. After an incubation period of 10 min at 4°C in the dark, 30 µl of MACS buffer and 20 µl of Anti-Biotin MicroBeads per 10⁷ cells were added, followed by another incubation for 15 min at 4°C in the dark. Cells were washed with MACS buffer (300 x g, 20°C, 10 min), supernatants were discarded and pellets were resuspended in 500 µl of MACS buffer per 10⁸ cells. LS Columns were placed in a MACS separator and washed with 3 ml of MACS buffer. Cell suspension was added onto the columns and the columns were washed three times with 3 ml of MACS buffer. The effluent fraction contained the non-labelled CD4⁺ T cells. Purity of CD4⁺ T cells was always higher than 95%.

2.2 Cell culture

Cells were incubated in a WTB Binder Incubator (Binder GmbH, Tuttlingen, Germany) at 37°C in 5% carbon dioxide atmosphere. PDCs were cultured in AIM-V (Adoptive Immunotherapy Media) medium (Life Technologies, Carlsbad, CA, USA) with or without IL-3 (R&D Systems, Minneapolis, MN, USA). IL-3 was supplemented at a final concentration of 10 ng/ml. PDCs were seeded either in 96-well flat- or round-bottom plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 1×10^5 cells per well at a total volume of 200 μ l. Except for spinning disc confocal microscopy and MLR experiments, incubation of pDCs generally lasted 24 hours.

PDCs were stimulated with various concentrations of vaccines against inactivated polio virus (*IPV Mérieux*), yellow fever (*Stamaril*), measles (*Masern-Impfstoff Mérieux*), rubella (*Röteln-Impfstoff HDC Mérieux*, all Sanofi Pasteur MSD, Leimen, Germany) and tick-borne encephalitis (*FSME immun Erwachsene*, Baxter, Deerfield, IL, USA). The vaccines against polio and tick-borne encephalitis contain inactivated viruses whereas the vaccines against yellow fever, measles and rubella consist of attenuated live viruses. PDCs were alternatively treated with the pure, live attenuated measles virus (Edmonston B strain), kindly provided by C. Beltinger and colleagues (University Medical Center Ulm, Department of Pediatrics). This virus strain was produced in Vero cells (a cell-line derived from the kidney of an African green monkey) and used at multiplicities of infection (MOIs) ranging from 0.01 to 2.

Class B oligodeoxynucleotide (ODN) 2006 (Pfizer, New York, NY, USA) containing a cytosine-phosphate-guanosine (CpG) motif with the sequence 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' was used as additional stimulus at a concentration of 2.5 μ g/ml.

T cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% of fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA), 2 mM L-glutamine (Life Technologies, Carlsbad, CA, USA), 1 mM sodium pyruvate and 10 mM HEPES buffer (both Biochrom AG, Berlin, Germany).

2.3 Surface staining for fluorescence-activated cell sorting (FACS) analysis

Antibodies against CD123, CD86, CD54, HLA-DR, lineage-1 (lin-1) (all Becton Dickinson, Franklin Lakes, NJ, USA) and BDCA-2 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used in different combinations. Phycoerythrin (PE), PE tandem with cyanin dye (PECy) 5, PECy7, peridinin chlorophyll protein tandem with cyanin dye (PerCPCy) 5.5, allophycocyanin (APC), Pacific Blue and fluorescein isothiocyanate (FITC) were conjugated as fluorescent dyes to the different antibodies.

PDCs were harvested into 5 ml FACS tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and in general 3 µl of each fluorescent antibody were added except for lin-1 (1 µl) and BDCA-2 (2 µl). After an incubation period of 10 min at RT in the dark, cells were washed with 2 ml of PBS (350g, 20°C, 7 min). Supernatants were aspirated up to a remaining volume of 200 µl and fluorescence was measured either on a FACS Scan, FACS Calibur or FACS LSRII (all Becton Dickinson, Franklin Lakes, NJ, USA). In cases of pDC-purity lower than 90 %, pDCs were gated as lineage⁻, CD123⁺ cells. Expression of surface molecules was quantified as average mean fluorescence intensity (MFI). Analysis of FACS data was generally performed by using FlowJo software 8.7.1 (TreeStar, Ashland, OR, USA).

2.4 Intracellular staining for granzyme B

Intracellular granzyme B staining was preceded by the incubation of pDCs with Brefeldin A (Epicentre Technologies, Madison, WI, USA) at a concentration of 1 µg/ml for four hours in order to disrupt exocytosis and accumulate intracellularly produced proteins. Cells were harvested in 5 ml FACS tubes and staining for surface molecules was performed as described above. After washing and aspirating the supernatants, pDCs were fixed (15 min, RT, in the dark) with 100 µl of fixation medium consisting of PBS and 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) and washed with PBS (350 x g, 20°C, 7 min). Supernatants were aspirated and cells were permeabilised by 100 µl of permeabilisation medium (PBS, 0.25% saponin (Sigma-Aldrich, St. Louis, MO, USA), filtered sterile) for 15 min at RT in the dark. 2 µl of FcR Blocking Reagent (Miltenyi Biotec GmbH,

Bergisch Gladbach, Germany) were added, followed by an incubation period of 10 min at RT in the dark. PDCs were stained with 2 µl of anti-human GrB-PE-conjugated antibody (Sanquin, Amsterdam, The Netherlands) or 2 µl of IgG₁ isotype control antibody (R&D Systems, Minneapolis, MN, USA) for 15 min at RT in the dark prior to another washing step (350 x g, 20°C, 7 min). PDCs were finally analysed by flow cytometry. Intracellular levels of GrB were quantified as average MFI of GrB.

2.5 Annexin V - propidium iodide staining

PDCs were harvested into 5 ml FACS tubes and surface staining as described above was performed if necessary. Cells were washed twice with 3 ml of 1 x Annexin Binding Buffer (Sterofundin (B. Braun Melsungen AG, Melsungen, Germany), 0.01 M Hepes buffer) at 350 x g at 20°C for 7 min, supernatants were aspirated and 5 µl of FITC or APC-conjugated Annexin V (both Becton Dickinson, Franklin Lakes, NJ, USA) were added to 100 µl of cell suspension. After an incubation period of 15 min at RT in the dark, cells were washed with 1 x Annexin Binding Buffer (350 x g, 20°C, 7 min). Supernatants were aspirated again and propidium iodide (PI) solution (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2.5 µg/ml was added. Immediate assessment of cell viability via flow cytometry followed. Cell viability was quantified as percentage of annexin V⁻, PI⁻ cells.

2.6 Enzyme-linked immunosorbent assay

PDCs were stimulated as described and after centrifugation of the cells (250 x g, 10 min, RT), 150 µl of supernatants were collected, split and stored at -80°C. 75 µl were analysed via GrB enzyme-linked immunosorbent assay (ELISA) and IFN-α ELISA.

2.6.1 Granzyme B ELISA

Plates for GrB ELISA were assembled by using Nunc-immuno Modules (Thermo Electron LED GmbH, Langenselbold, Germany). ELISAs were performed according to the manufacturer's protocol using Human Granzyme B ELISA kit

(Mabtech AB, Nacka Strand, Sweden). Plates were coated with 100 µl of monoclonal anti-GrB antibody (mAb GB10, 2 µg/ml final concentration), covered with a plate sealer and incubated overnight at 4°C. The plates were washed twice with 200 µl of PBS and blocked with 200 µl of incubation buffer (PBS, 0.05 % Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), 0.1 % BSA) for one hour at RT. After blocking, plates were washed again five times with 200 µl of wash buffer (PBS, 0.05 % Tween). Human Granzyme B standard was diluted using incubation buffer to a final concentration of 5000 pg/ml (standard 1) and subsequently diluted 1:2 to finally 78.125 pg/ml (standard 7). 100 µl of standards, blanks (incubation buffer) or 1:2 diluted samples were placed in each well, plates were covered and incubated for two hours at RT. After five further washing steps, 100 µl of biotinylated monoclonal anti-GrB antibody (mAb GB11, 1 µg/ml) were added, followed by another incubation period (1h, RT, plates covered). Plates were washed five times before the addition of 100 µl of streptavidin-conjugated horseradish peroxidase (HRP) diluted 1:1000 in incubation buffer for another hour at RT with plates being covered. After five washing steps, plates were incubated with 100 µl of a 1:1 mixture of colour reagent a (stabilised peroxide solution) and b (stabilised chromogen solution) from Substrate Reagent Pack (R&D Systems, Minneapolis, MN, USA) for 20 min at RT in the dark without plate sealer. Absorption was immediately measured on a Mithras LB 940 plate reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) at 450 nm after stopping the reaction with 50 µl of 2N sulphuric acid (Sigma-Aldrich, St. Louis, MO, USA).

2.6.1 Interferon-α ELISA

Interferon-α ELISA was performed using Human Interferon Alpha Multi-subtype ELISA KIT (PBL Biomedical Laboratories, Piscataway, NY, USA) according to the manufacturer's protocol. 100 µl of blanks, standards and 1:2 diluted samples per well were placed into pre-coated 96-well ELISA plates and incubated for one hour at RT. Human IFN-α Standard was diluted with dilution buffer to final concentrations ranging from 5000 pg/ml (standard 1) to 78.125 pg/ml (standard 7). After washing once with 250 µl of wash buffer (1:20 diluted wash solution), plates were incubated with 100 µl of diluted antibody concentrate, containing polyclonal anti-human IFN-α antibodies (1h, RT, plates covered). Plates were washed three times and 100 µl of anti-IgG-conjugated HRP solution were added for another hour

at RT. Before addition of the HRP substrate tetramethylbenzidine for 15 min at RT in the dark, plates were washed again four times. The reaction was stopped with 100 μ l of stop solution and absorbance was immediately measured at 450 nm on a Mithras LB 940 plate reader.

2.7 Mixed lymphocyte reaction

For MLR experiments, pDCs were isolated using the Plasmacytoid dendritic cell isolation kit human as described. PDCs were prestimulated with different antiviral vaccines in AIM-V medium in the presence of IL-3 for 48h in 96-well flat-bottom plates. Cell densities for MLR experiments varied between 1×10^5 and $1,5 \times 10^5$ pDCs per well among different experiments. After preincubation, pDCs were washed once with PBS (250 x g, 7 min, 20°C) and counted. CD4⁺ T cells were isolated as described above. Before cocultivation of T cells and pDCs, T cells were stained with carboxyfluoresceindiacetate succinimidyl ester (CFSE) using the CellTrace CFSE Proliferation Kit (Life Technologies, Carlsbad, CA, USA) according to the slightly modified manufacturer's protocol. Therefore, isolated T cells were counted, spun down (350 x g, 7 min, 20°C) and resuspended in 400 μ l of PBS for each 10^7 cells. T cells were incubated with CFSE solution at a final concentration of 2.5 μ M for 10 min in the incubator. After blocking for 10 min in medium (RPMI 1640, 10 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer), cells were washed three times with PBS (400 x g, 7 min, 20°C) and counted. Finally, 2×10^5 T cells were cocultured with pDCs in 96-well round bottom plates at different pDC to T cell ratios, namely 1:50, 1:250 and 1:1250. T cells alone were stimulated with 0.5 μ l of anti-CD3 and anti-CD28 beads (Dynabeads CD3/CD28, Life Technologies, Carlsbad, CA, USA) as positive control for T cell proliferation. After six days, cells were transferred to 5 ml FACS tubes and surface staining for CD3 was performed as described above. After additional staining with 7-amino-actinomycin D (7-AAD, Merck KGaA, Darmstadt, Germany) at a final concentration of 2.85 μ M, cells were analysed via flow cytometry on a FACS LSR II. Proliferation of CD3⁺, 7-AAD⁻ T cells was assessed as percentage of CFSE^{low} cells.

2.8 Spinning disc confocal microscopy

For spinning disc confocal microscopy, pDCs were isolated using the Plasmacytoid dendritic cell isolation kit human and cultured in IL-3-containing AIM-V medium in the presence or absence of tick-borne encephalitis vaccine (TBEV) at a concentration of 100 ng/ml for 60 hours. CD4⁺ T cells were purified as described and cultured in growth medium for 24 hours. 10⁵ T cells were seeded on ibiTreat chamber slides (ibidi GmbH Integrated BioDiagnostics, Martinsried, Germany). After an immobilisation period of one hour, cells were washed three times with PBS, stained with CellMask Deep Red membrane stain (Life Technologies, Carlsbad, CA, USA) at a final concentration of 0.5 µg/ml for 5 min and washed again three times with PBS.

25 µl of GranToxiLux fluorogenic GrB substrate (OncoImmunin Inc, Gaithersburg, MD, USA) were added to 10⁵ pDCs in 75 µl of AIM-V medium. PDCs were directly added to immobilised T cells in chamber slides and cocultured for several hours. The spinning disc confocal microscope used to perform fluorescence imaging was assembled from individual components including a Confocal scanner unit 10 scan head (Yokogawa Electric Corporation, Tokyo, Japan), an inverted microscope (Axio Observer; Carl Zeiss AG, Oberkochen, Germany) with oil-immersion objective (UPlanSApo 60x1.35, Olympus, Tokyo, Japan), environmental control (PeCon GmbH, Erbach, Germany), an image-splitting unit (OptoSplit II, Cairn Research Limited, Kent, UK) and an electron-multiplying charge-coupled device camera (DV-887, Andor Technologies, Belfast, Northern Ireland). Images were acquired using Andor iQ 1.6 software (Andor Technologies, Belfast, Northern Ireland) and image processing was done with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.9 Statistics

Data is expressed as means and aberrations as standard errors of mean. Statistical significance was tested by applying a paired two-tailed student's t-test. Results were considered significant with p-values < 0.05 and highly significant with p-values < 0.005.

3. Results

3.1 Commonly used antiviral vaccines reduce granzyme B production and secretion of plasmacytoid dendritic cells

In order to test the hypothesis that commonly used antiviral vaccines lead to a more immunogenic phenotype in pDCs with lower levels of GrB, the effects of the vaccines on GrB production and secretion by pDCs were evaluated first via flow cytometry and ELISA. Intracellular levels of GrB were analysed by quantification of the mean fluorescence intensity (MFI) of GrB in FACS data. Vaccines included in this study were inactivated polio vaccine (IPV), yellow fever vaccine (YFV), measles vaccine (MV), tick-borne encephalitis vaccine (TBEV) and rubella vaccine (RV).

The stimulation of pDCs with various dilutions of polio vaccine decreased the number of GrB⁺ cells (figure 1A). Low amounts of GrB were already detectable without stimulation on the day of isolation (Day 0) and strongly increased after culturing of pDCs in IL-3-containing medium. Stimulation with polio vaccine significantly reduced the production of GrB in a dose-dependent manner. The relative MFI of GrB in pDCs decreased up to 77.7 ± 4.8 % upon stimulation with the highest concentration of polio vaccine and IL-3 compared to IL-3-only-treated cells.

GrB levels were substantially lower when pDCs were cultured without supplementation of IL-3. The relative MFI of GrB was 31.3 ± 7.7 % in pDCs treated with medium only compared to IL-3-stimulated cells. However, stimulation with polio vaccine led to a reduction of intracellular GrB levels similarly to the conditions with IL-3 (figure 1B).

In order to confirm these findings, the supernatants of vaccine-stimulated pDCs were analysed via GrB ELISA. The secretion of GrB also strongly declined after stimulation with polio vaccine in a concentration-dependent manner (figure 1C).

As the viability of pDCs as well as the intracellular and secreted amounts of GrB were markedly lower when pDCs were cultured in growth medium without IL-3, all subsequent data refer to experiments with supplementation of IL-3.

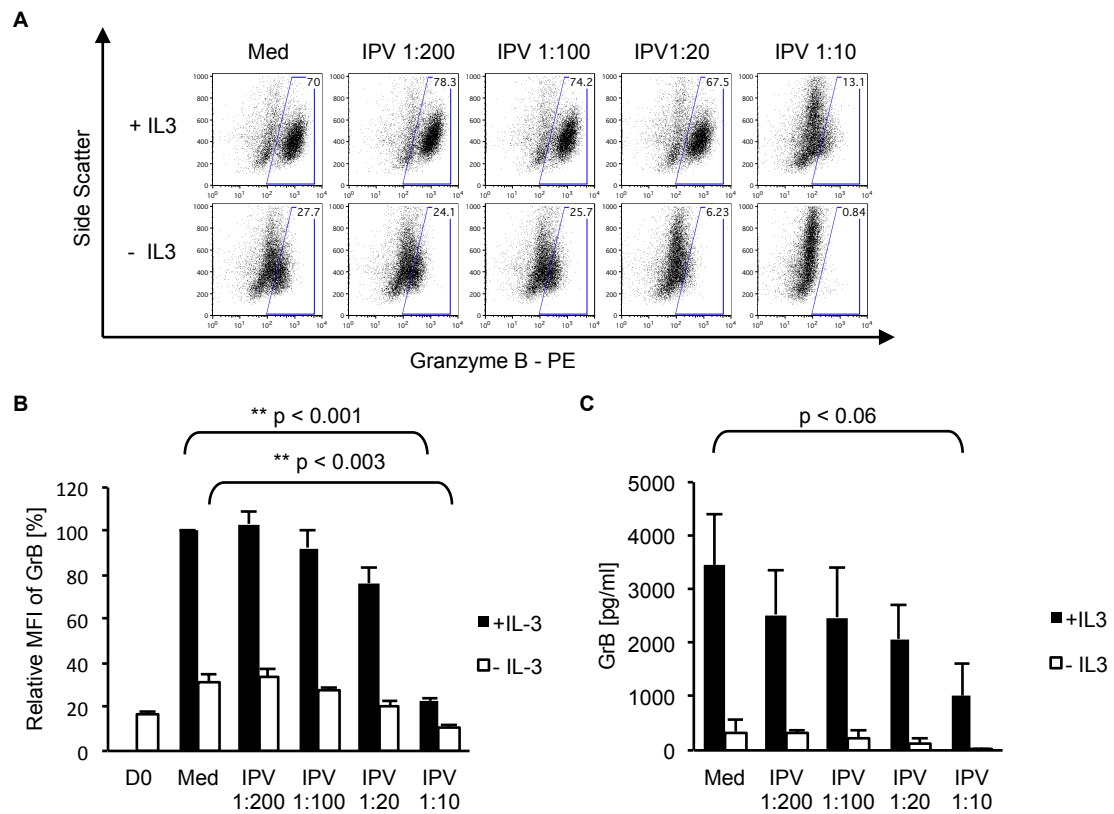


Figure 1. Inactivated polio vaccine (IPV) inhibits production and secretion of granzyme B (GrB) in plasmacytoid dendritic cells (pDCs).

PDCs were isolated as described, incubated for 24 hours in AIM-V medium (Med) supplemented with or without interleukin (IL)-3 (10 ng/ml) and stimulated with different concentrations of IPV as indicated. A+B) PDCs were stained for intracellular GrB as described. A) Representative dot plots of one experiment are shown. Gates indicate the percentage of GrB⁺ pDCs. B) Bar graphs show average mean fluorescence intensity (MFI) of GrB normalised to IL-3 from at least 6 (with IL-3) or 3 (without IL-3) different donors. Day 0 (D0) was stained immediately after isolation of pDCs without stimulation. C) Enzyme-linked immunosorbent assay of supernatants collected after stimulation of pDCs for 24 hours. Diagram shows mean concentration of GrB in the supernatant from at least 5 (with IL-3) or 3 (without IL-3) different donors. Error bars represent standard error of mean, ** highly significant results with p values < 0.005. PE: Phycoerythrin.

To validate the data found for polio vaccine, several other antiviral vaccines were tested. Basically, all analysed vaccines showed the same tendency concerning their potential to reduce intracellular GrB levels in pDCs in a dose-dependent manner, however to different extents.

While rubella vaccine exerted almost no effect on intracellular GrB, yellow fever, measles and tick-borne encephalitis vaccine significantly downregulated GrB production to a similar degree (figure 2 A-D). Compared to the other vaccines, polio vaccine induced the strongest reduction of intracellular GrB levels (figure 2E).

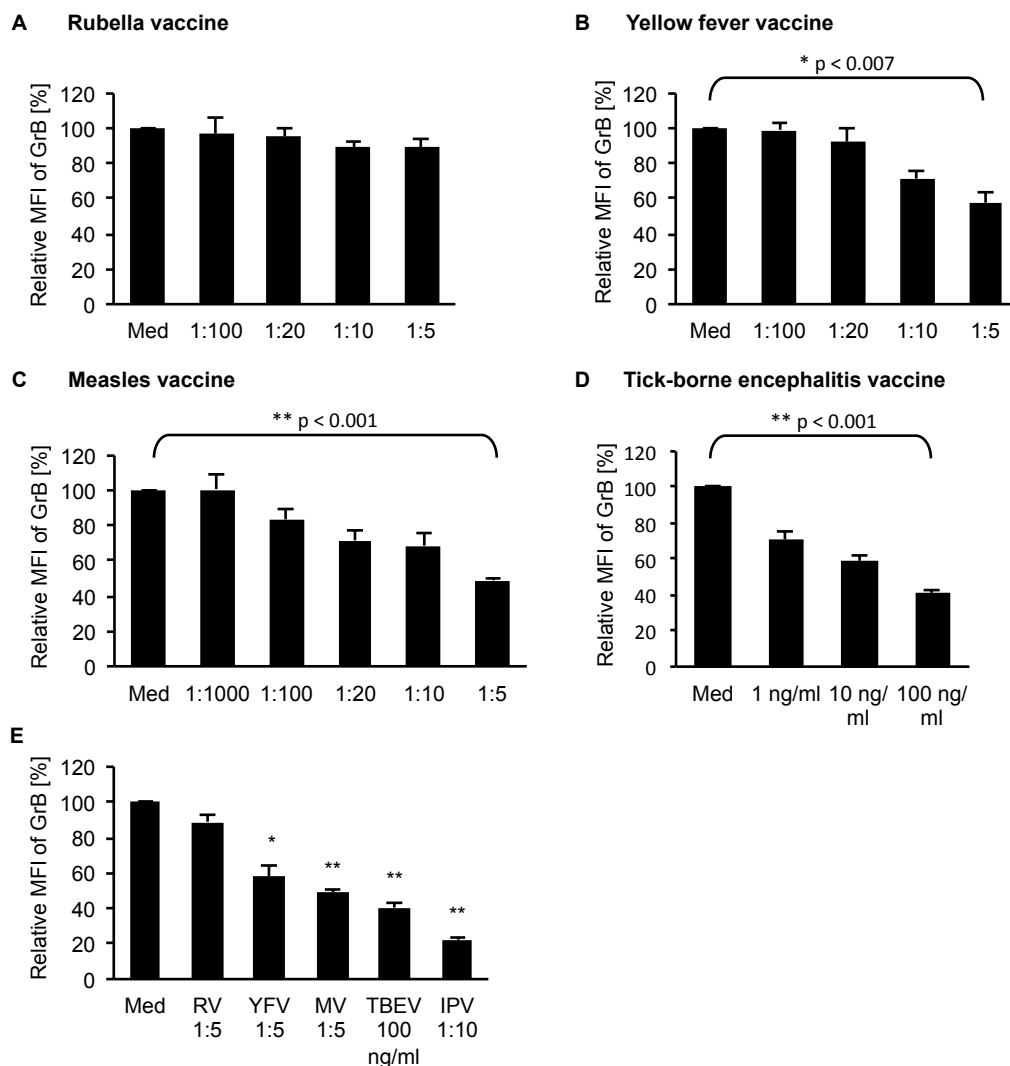


Figure 2. Dose-dependent effects of antiviral vaccines on granzyme B (GrB) production in plasmacytoid dendritic cells (pDCs).

PDCs were isolated as described, incubated for 24 hours in interleukin-3-containing medium (Med) and stimulated with different dilutions of antiviral vaccines as indicated. PDCs were stained for intracellular GrB as described and analysed via flow cytometry. A-D) Serial dilutions of antiviral vaccines are shown. Bar graphs represent average mean fluorescence intensity (MFI) of GrB normalised to Med from at least 3 (A), 4 (B), 5 (C) or 8 (D) different donors. E) Bar graphs show average MFI of GrB of the highest vaccine concentrations used normalised to Med. Error bars indicate standard error of mean, * significant results with p values < 0.05, ** highly significant results with p values < 0.005. RV: rubella vaccine, YFV: yellow fever vaccine, MV: measles vaccine, TBEV: tick-borne encephalitis vaccine, IPV: inactivated polio vaccine.

In line with the results observed for intracellular GrB levels, GrB secretion by pDCs also decreased in a dose-dependent manner after stimulation with the other tested vaccines. Moderate effects on GrB secretion could be observed upon stimulation with yellow fever or rubella vaccine, whereas measles vaccine – like polio vaccine described above – strongly diminished extracellular amounts of GrB. In case of TBEV-stimulated pDCs at a concentration of 100 ng/ml, GrB secretion was nearly completely abrogated (figure 3).

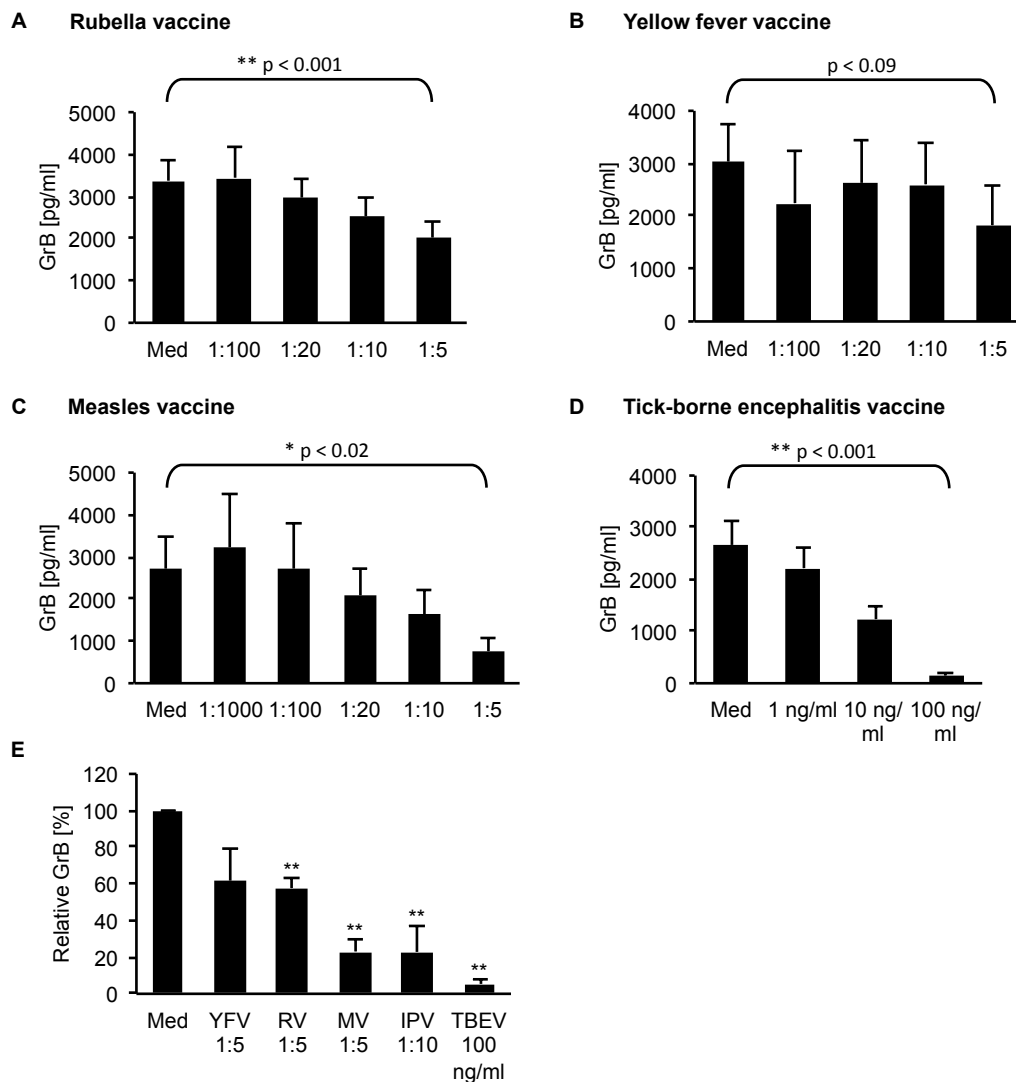


Figure 3. Dose-dependent effects of antiviral vaccines on granzyme B (GrB) secretion by plasmacytoid dendritic cells (pDCs).

PDCs were isolated as described, incubated for 24 hours in interleukin-3-containing medium (Med) and stimulated with different dilutions of antiviral vaccines as indicated. Supernatants were collected and analysed via GrB enzyme-linked immunosorbent assay (ELISA). A-D) Serial dilutions of different antiviral vaccines are shown. Bar graphs represent mean concentration of GrB in the supernatants from at least 5 (A), 3 (B), 6 (C) or 9 (D) different donors. E) Bar graphs show mean concentration of GrB in the supernatants of the highest vaccine concentrations used normalised to Med. Error bars indicate standard error of mean, * significant results with p values < 0.05, ** highly significant results with p values < 0.005. RV: rubella vaccine, YFV: yellow fever vaccine, MV: measles vaccine, TBEV: tick-borne encephalitis vaccine, IPV: inactivated polio vaccine.

3.2 Tick-borne encephalitis vaccine induces substantial interferon- α secretion by plasmacytoid dendritic cells

IFN- α is a strong proinflammatory cytokine that is produced in high quantities during viral infections by pDCs. Thus, the potential of antiviral vaccines to induce IFN- α secretion by pDCs was determined in a further step. For that purpose, pDCs were stimulated for 24 hours with different antiviral vaccines and the supernatants were analysed via IFN- α ELISA.

Unstimulated pDCs secreted only very low to not detectable levels of IFN- α . Polio, yellow fever, measles and rubella vaccines failed to stimulate IFN- α secretion in pDCs. However, tick-borne encephalitis vaccine caused a significant IFN- α response. The mean IFN- α concentration in the supernatants of unstimulated pDCs was 26.3 pg/ml and rose to 3190.6 ng/ml upon stimulation with TBEV at a final concentration of 10 ng/ml (Figure 4).

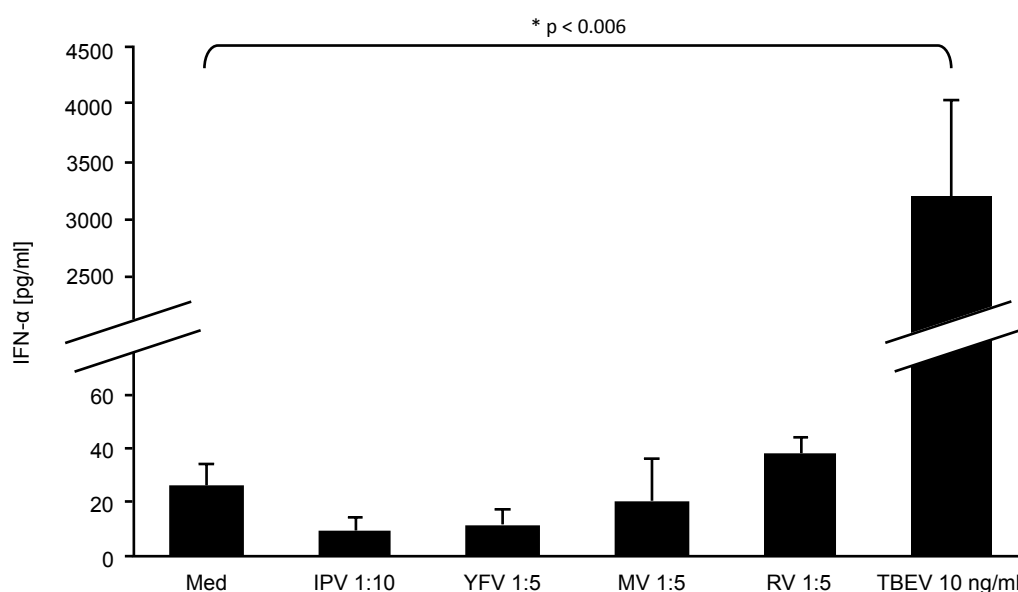


Figure 4. Effects of antiviral vaccines on interferon (IFN)- α secretion by plasmacytoid dendritic cells (pDCs).

PDCs were cultured in AIM-V medium (Med) supplemented with interleukin-3 (10 ng/ml) and stimulated with different antiviral vaccines at concentrations as indicated. Supernatants were collected after 24 hours and analysed via enzyme-linked immunosorbent assay (ELISA). Bar graphs show mean IFN- α concentration from 11 (Med), 8 (IPV), 5 (YFV), 5 (MV), 4 (RV) and 9 (TBEV) different donors. Error bars represent standard error of mean, * significant result with p value < 0.05. IPV: inactivated polio vaccine, YFV: yellow fever vaccine, MV: measles vaccine, RV: rubella vaccine, TBEV: tick-borne encephalitis vaccine.

3.3 Pure measles virus reduces intracellular granzyme B levels in plasmacytoid dendritic cells and induces granzyme B and interferon- α secretion

As the different antiviral vaccines always contain various admixtures whose influence can be difficult to quantify or rule out, the influence of pure measles virus on pDCs was also explored. The measles virus Edmonston B strain used in this set of experiments differed from the strain of the vaccine (Schwarz strain).

Upon stimulation with increasing multiplicities of infection (MOIs) of measles virus, the number of GrB⁺ pDCs declined (figure 5A). Additionally, the amount of intracellular GrB in pDCs significantly decreased in a dose-dependent manner (figure 5B).

Interestingly, in contrast to the effects of the measles vaccine described above, the pure Edmonston B measles virus showed a tendency to enhance the amount of secreted GrB with increasing MOIs compared to unstimulated pDCs (figure 5C).

Furthermore, the measles vaccine did not exert noticeable influence on pDC-derived IFN- α , whereas the pure measles virus induced a significant IFN- α response in pDCs. The mean IFN- α secretion augmented from 9.5 pg/ml by untreated pDCs to a maximum of 2246.6 pg/ml upon stimulation with measles virus at a MOI of 1 (figure 5D).

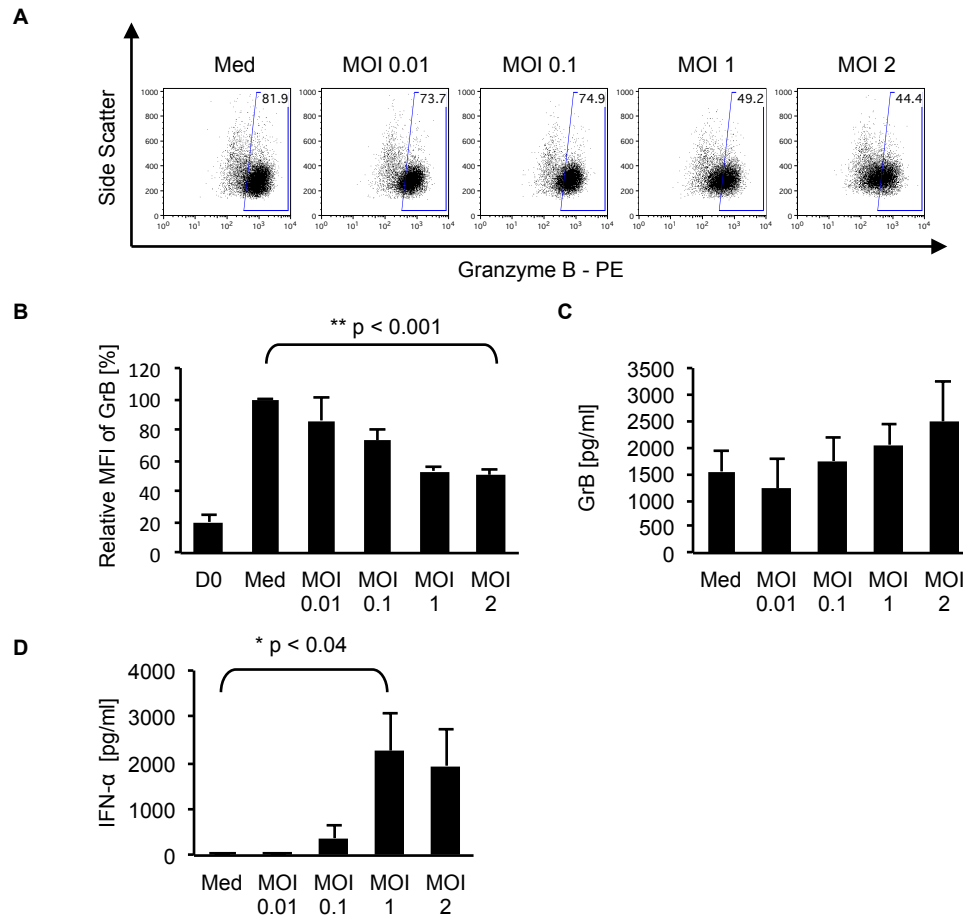


Figure 5. Effects of pure measles virus on plasmacytoid dendritic cell (pDC)-derived granzyme B (GrB) and interferon (IFN)- α .

PDCs were isolated as described and incubated for 24 hours with different multiplicities of infection (MOIs) of the Edmonston B measles virus as indicated. All conditions were cultured in AIM-V medium (Med) supplemented with interleukin-3 (10 ng/ml). A+B) PDCs were stained for intracellular GrB and analysed via flow cytometry. A) Representative dot plots of one experiment are shown. Gates indicate percentage of GrB⁺ pDCs. B) Bar graphs show average mean fluorescence intensity (MFI) of GrB normalised to Med from at least five different donors. Day 0 (D0) was stained immediately after isolation of pDCs without stimulation. C) GrB enzyme-linked immunosorbent assay (ELISA) of supernatants collected after stimulation of pDCs for 24 hours. Diagram shows mean concentration of GrB in the supernatant of at least six different donors. D) IFN- α ELISA of supernatants of at least four different donors. Error bars represent standard error of mean, * significant results with p values < 0.05, ** highly significant results with p values < 0.005. PE: Phycoerythrin.

3.4 Antiviral vaccines weakly modulate surface molecule expression of plasmacytoid dendritic cells

After evaluation of the capacity of pDCs to produce and secrete GrB and IFN- α upon stimulation with antiviral vaccines, the influence of these vaccines on the phenotype of pDCs was examined. Activation and maturation of pDCs generally lead to the upregulation of distinct surface molecules. For that purpose, FACS analysis of the expression of three exemplary surface molecules, namely HLA-DR as a molecule for antigen presentation, CD54 as an intercellular adhesion molecule and CD86 as a costimulatory protein, was carried out.

Polio, yellow fever and measles vaccine did not substantially alter the expression of HLA-DR, CD54 and CD86. Solely tick-borne encephalitis vaccine significantly upregulated CD86. The relative MFI of CD86 was 2.48 ± 0.75 fold higher after stimulation of pDCs with TBEV at a concentration of 100 ng/ml compared to unstimulated pDCs (figure 6).

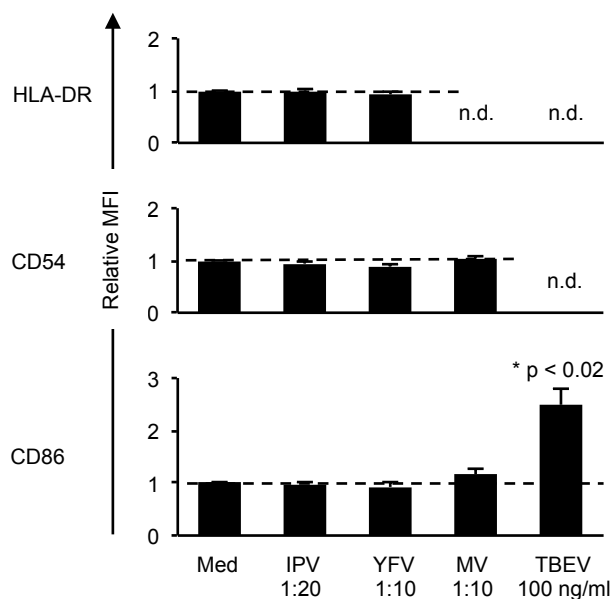


Figure 6. Effects of antiviral vaccines on phenotype of plasmacytoid dendritic cells (pDCs).

Purified pDCs were incubated for 24 hours in interleukin-3-containing AIM-V medium (Med) and stimulated with different antiviral vaccines at concentrations as indicated. Surface molecule expression of cluster of differentiation (CD)86, CD54 and human leukocyte antigen (HLA)-DR were analysed by flow cytometry. Bar graphs represent average mean fluorescence intensities (MFIs) normalised to medium of at least three different donors. Error bars indicate standard error of mean, * significant results with p values < 0.05. IPV: inactivated polio vaccine, YFV: yellow fever vaccine, MV: measles vaccine, TBEV: tick-borne encephalitis vaccine, n.d.: not determined.

3.5 Antiviral vaccines do not significantly induce cell death in plasmacytoid dendritic cells

For FACS analysis of intracellular GrB in pDCs, gating on morphological viable cells was performed. However, these morphological viable cells might still differ in cell viability upon stimulation with antiviral vaccines compared to untreated pDCs. Thus, annexin V - PI stainings to assess cell viability were carried out in order to exclude the possibility that the decrease of intracellular GrB levels in pDCs after stimulation is due to cytotoxic effects of the vaccines. Cell viability was quantified as percentage of annexin V and PI negative cells.

Cell viability of morphological viable pDCs was not significantly decreased by antiviral vaccines. Only the highest concentration of polio vaccine (1:10 dilution) led to a moderate reduction of the annexin V⁻ PI⁻ fraction of pDCs (figure 7). Viability assessment of all cells without pre-gating on morphological viable cells revealed similar results (data not shown).

These results document that the observed effects of antiviral vaccines on pDCs are not caused by possible cytotoxic effects of the vaccines.

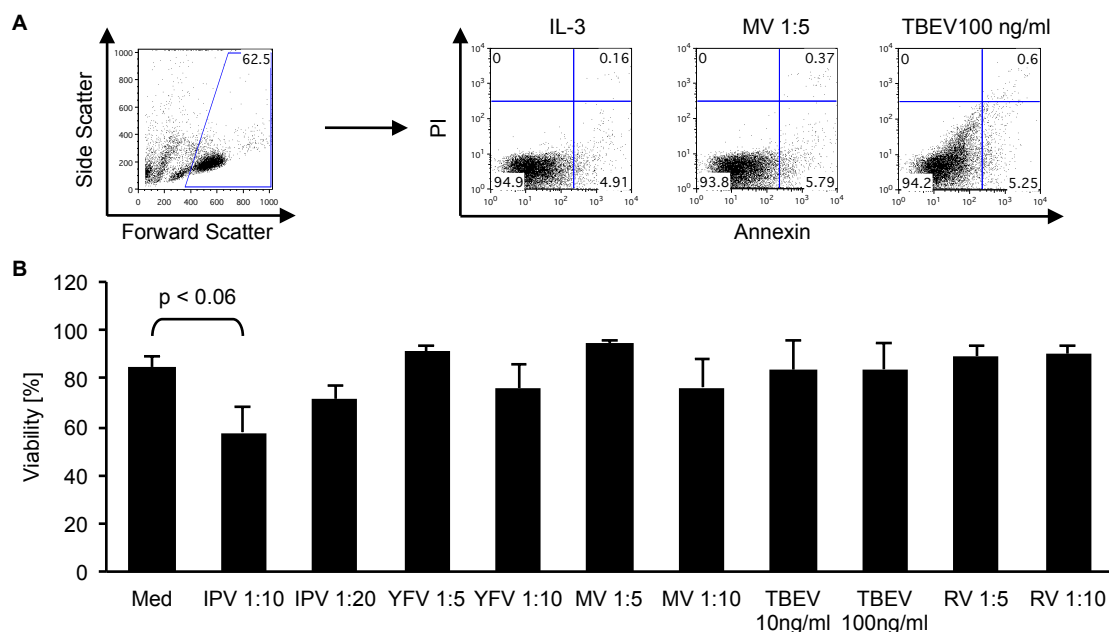


Figure 7. Effects of antiviral vaccines on cell viability of plasmacytoid dendritic cells (pDCs).

Purified pDCs were incubated in interleukin (IL)-3-containing AIM-V medium (Med) and stimulated with different antiviral vaccines diluted as indicated for 24 hours. Cell viability was assessed performing annexin V – propidium iodide (PI) staining. PDCs were analysed via flow cytometry. A) Representative dot plots are shown. Left single dot plot demonstrates gating on morphological viable cells. Right group of dot plots shows annexin V - PI stainings for two exemplary vaccines. B) Bar graphs show average percentage of annexin V – PI negative cells of morphological viable pDCs from at least three different donors. Error bars indicate standard error of mean. MV: measles vaccine, TBEV: tick-borne encephalitis vaccine, IPV: inactivated polio vaccine, YFV: yellow fever vaccine, RV: rubella vaccine.

3.6 Adjuvant aluminium hydroxide does not influence IFN- α secretion and weakly affects production and secretion of granzyme B in plasmacytoid dendritic cells

Many commonly used vaccines contain adjuvants in order to increase their immunogenicity. One frequently used adjuvant is aluminium hydroxide which is also part of the tick-borne encephalitis vaccine. To investigate whether the influence of TBEV on GrB and IFN- α is primarily contributed to the virus particles or rather to effects of the adjuvant, pDCs were stimulated with aluminium hydroxide only and subsequently, intracellular GrB production and GrB secretion were analysed and compared with TBEV-stimulated pDCs.

Intracellular GrB levels in pDCs were only weakly modulated by adjuvant aluminium hydroxide compared to untreated cells. However, a significant reduction of GrB production could be observed after stimulation of pDCs with TBEV compared to the adjuvant (figure 8A).

Aluminium hydroxide led to a moderate, dose-dependent inhibition of GrB secretion whereas stimulation with TBEV strongly augmented this inhibitory effect (figure 8B).

Concerning IFN- α secretion by pDCs, there is a substantial difference between TBEV and the adjuvant. While TBEV induced a pronounced IFN- α response after 24 hours as reported above, aluminium hydroxide did not influence IFN- α secretion at all (figure 8C).

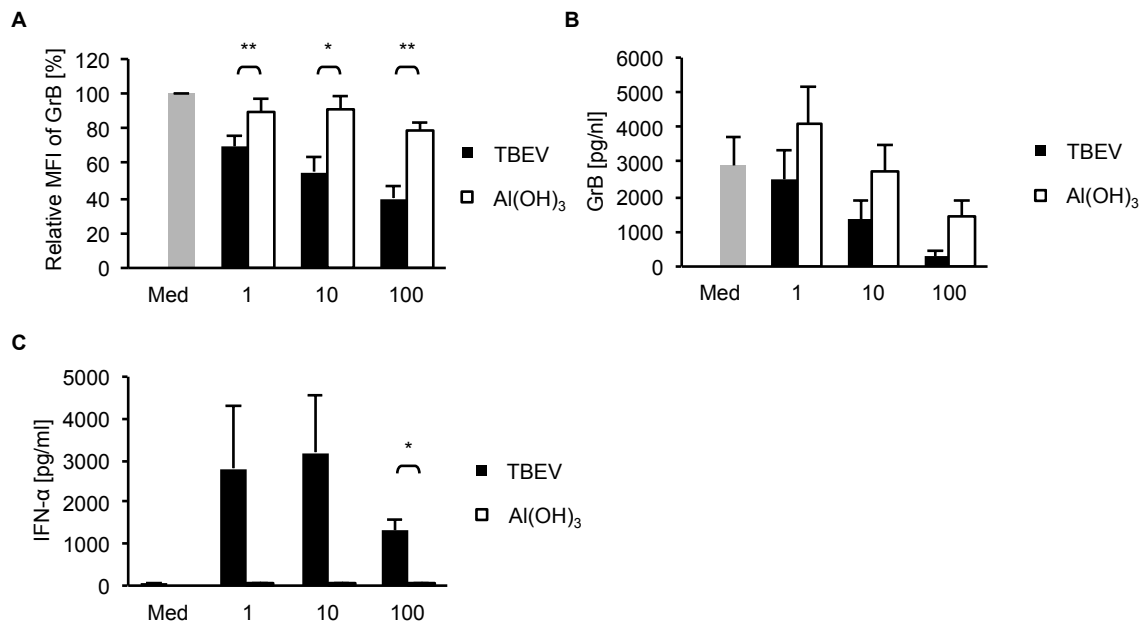


Figure 8. Effects of adjuvant aluminium hydroxide (Al(OH)₃) on plasmacytoid dendritic cells (pDCs).

Purified pDCs were cultured in interleukin-3-containing AIM-V medium (Med) and stimulated with different concentrations of tick-borne encephalitis vaccine (TBEV) or Al(OH)₃ for 24 hours. The concentration of TBEV is indicated in ng/ml. The concentration of Al(OH)₃ was adjusted to the vaccine dose (e.g. 100 ng/ml of TBEV correspond to 14.6 µg/ml Al(OH)₃). A) PDCs were stained for intracellular granzyme B (GrB) and analysed via flow cytometry. Bar graphs represent average mean fluorescence intensity (MFI) of GrB normalised to medium of at least four different donors. B) GrB enzyme-linked immunosorbent assay (ELISA) of supernatants collected after stimulation of pDCs for 24 hours. Diagram shows mean concentration of GrB from 3 (TBEV 1, 10) or 4 (all other conditions) different donors. C) Interferon (IFN)-α ELISA of supernatants after 24 hours of stimulation. Bar graphs represent mean IFN-α concentration from at least three different donors. Error bars indicate standard error of mean, * significant results with p values < 0.05, ** significant results with p values < 0.005.

3.7 Vaccine-stimulated pDCs cocultured with allogenic T cells modulate proliferative capacity of T cells

Activated pDCs gain the capacity to activate and stimulate other immune cells. One important cell type in the course of viral infections are CD4⁺ T cells that are primed and stimulated by dendritic cells.

As the results described above demonstrate that pDCs are differentially modulated by antiviral vaccines with altered surface molecule expression, lower levels of intracellular and secreted GrB and – in case of TBEV– marked secretion of IFN- α , coculture-studies of pDCs and T cells were performed to explore intercellular interactions between these two cell types.

First, mixed lymphocyte reaction experiments were carried out to analyse the potential of vaccine-stimulated pDCs to induce T cell proliferation. Negatively selected pDCs were prestimulated for 48 hours and then cocultured with allogenic, CFSE-labelled CD4⁺ T cells. Different pDC : T cell ratios ranging from 1:50 to 1:1250 were analysed. T cell proliferation was quantified after 6 days as fraction of CFSE^{low} T cells by means of flow cytometry.

The prestimulation of pDCs with antiviral vaccines weakly modulated the capacity of pDCs to induce T cell proliferation (figure 9). PDCs cultured in IL-3-containing medium provoked an average proliferation of T cells of 15.4 % at the highest pDC : T cell ratio that subsequently decreased at lower ratios. TBEV and MV as the strongest suppressors of GrB production and secretion failed to significantly augment the potential of pDCs to induce T cell expansion. However, substantial differences between individual donors could be observed (figure 9A). Interestingly, the stimulation of pDCs with the pure Edmonston B measles virus even at a low MOI of 0.1 significantly and nearly completely abrogated the potential of pDCs to induce proliferation. PDCs activated by TLR agonists like CpG ODN class B led to a pronounced average T cell proliferation of 35.6 % at the highest pDC : T cell ratio. T cells without any stimulus did not proliferate at all whereas activation of T cells by anti-CD3 and anti-CD28 dynabeads induced massive proliferation (figure 9C).

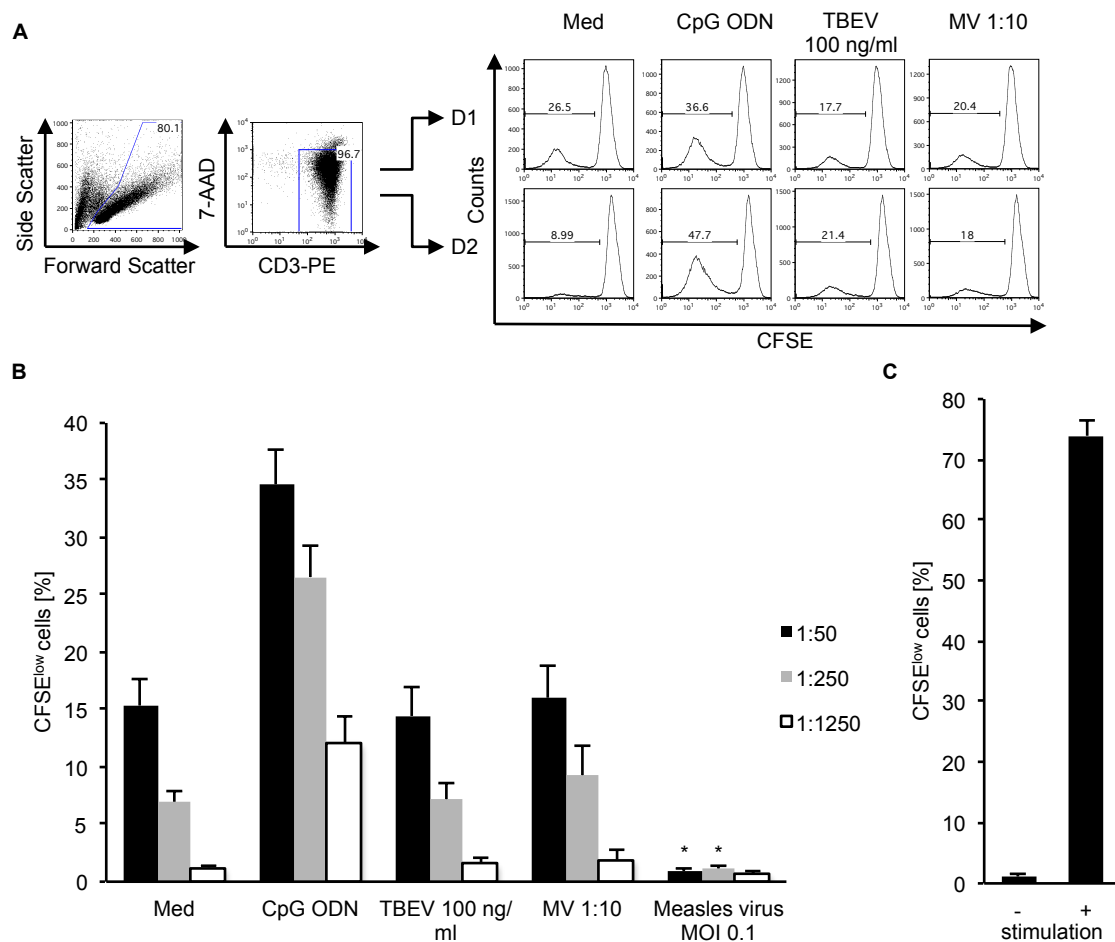


Figure 9. Effects of vaccine-stimulated plasmacytoid dendritic cells (pDCs) on T cell proliferation.

Isolated pDCs were cultured in interleukin (IL)-3 containing medium (Med) and prestimulated for 48 hours with different antiviral vaccines, cytosine-phosphate-guanosine oligodesoxynucleotides class B (CpG ODN, 2.5 µg/ml), or pure Edmonston B measles virus at a multiplicity of infection (MOI) of 0.1. PDCs were cocultured with purified, allogenic, carboxyfluoresceindiacetate succinimidyl ester (CFSE)-labelled CD4⁺ T cells for 6 days and T cell proliferation was assessed via flow cytometry. A) Left two dot plots demonstrate gating strategy on viable, 7-amino-actinomycin D (7-AAD)⁻, cluster of differentiation (CD)3⁺ T cells. Histogram plots show two individual combinations of pDC and T cell donors (D1, D2) from one experiment at a pDC : T cell ratio of 1:50. B) Bar graphs represent the mean percentage of CFSE^{low} T cells after coculture with pDCs from at least 6 pDC - T cell donor combinations. PDCs were prestimulated as indicated. C) Positive and negative control of T cell proliferation without pDC coculture. Bar graphs show mean percentage of CFSE^{low} T cells without any stimulus (-) or upon stimulation with 0.5 µl of CD3/CD28 beads (+). Error bars indicate standard error of mean, * significant results with p values < 0.05 compared to unstimulated pDCs (Med). TBEV: tick-borne encephalitis vaccine, MV: measles vaccine, PE: Phycoerythrin.

3.8 T cells in coculture with tick-borne encephalitis vaccine-stimulated plasmacytoid dendritic cells do not take up active granzyme B

In a second step, the pDC-T cell interactions were analysed on a cellular level by performing spinning disc confocal microscopy. PDCs were prestimulated for 60 hours with TBEV at a concentration of 100 ng/ml. Then, pDCs and T cells were cocultured and fluorescence images were acquired for up to six hours using time laps technique. Active granzyme B was visualised with GranToxiLux, a fluorogenic substrate of GrB.

Both TBEV-stimulated and untreated pDCs closely interacted with T cells. After approximately three hours, T cells that were coincubated with untreated pDCs became positive for active GrB while T cells that were cocultured with TBEV-treated pDCs remained GrB negative (figure 10).

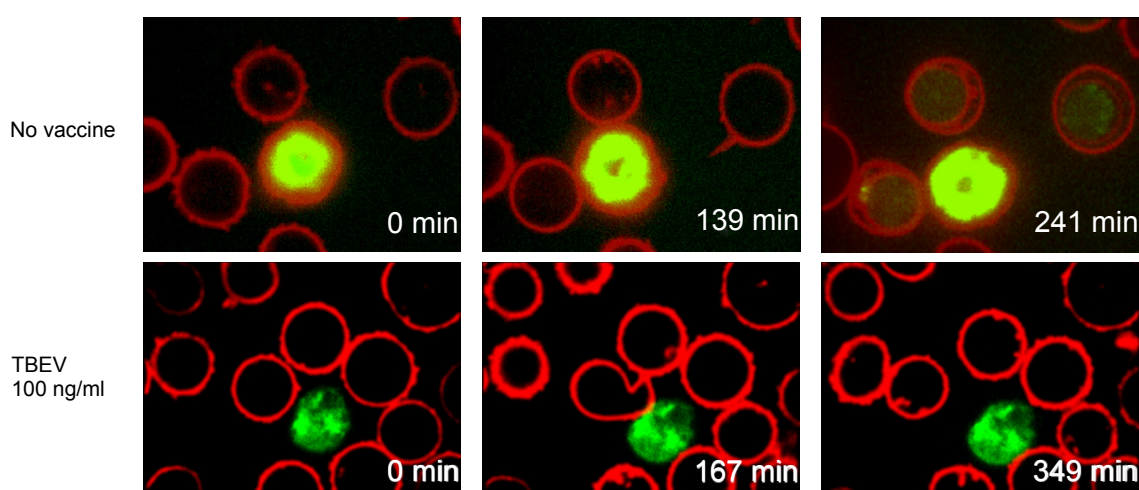


Figure 10. Plasmacytoid dendritic cells (pDCs) stimulated with tick-borne encephalitis vaccine (TBEV) do not transfer granzyme B (GrB) to T cells.

Purified pDCs were preincubated for 60 hours in AIM-V medium containing interleukin-3 (10 ng/ml) and either stimulated with TBEV (100 ng/ml) or not. Cluster of differentiation (CD)⁴ T cells were isolated as described and preincubated for 24 hours in growth medium. Cell membranes were stained with CellMask Deep red. Enzymatically active GrB was visualized with GranToxiLux fluorescent substrate. PDCs are displayed as bright green-flourescent cells. Weaker green fluorescence in the third image of the upper row indicates active GrB in T cells. Coculture of T cells and pDCs was analysed using spinning disc confocal microscopy. Images of two experiments are shown.

4. Discussion

4.1 Effects of antiviral vaccines on pDC-derived granzyme B

Nowadays it is generally accepted, that pDCs represent crucial mediators of innate and adaptive antiviral immunity [161, 164]. However besides their orchestrating antiviral role, growing evidence attributes several other features e.g. direct cytotoxic effects [25], tolerogenic properties [114] or GrB secretion [86] to pDCs. Despite their important antiviral function, the contribution of human pDCs to long-lasting immunity in the context of antiviral vaccination is barely analysed. Thus, the influence of commonly used antiviral vaccines on pDCs was examined in the present study to further explore underlying mechanisms of vaccine-induced immunity. Based on the previous study of our group that pDCs can be producers of large amounts of GrB, resulting in a diminished potential to stimulate T cell proliferation [86], a special focus was set on the influence of the vaccines on pDC-derived GrB.

This study demonstrated a general reduction of both intracellularly produced levels of GrB and secreted amounts of GrB by pDCs in a dose-dependent manner upon stimulation with several antiviral vaccines, namely vaccines against polio, measles, rubella, yellow fever and tick-borne encephalitis. However, the tested vaccines noticeably differed in their capacity to influence pDC-derived GrB. While TBEV, IPV and MV displayed rather strong effects, YFV and RV exerted less pronounced effects. Possible cytotoxic effects of the vaccines as cause of decreased expression of GrB could be ruled out, as the cell viability of pDCs did not significantly differ upon stimulation with vaccines compared to untreated cells. Some vaccines including TBEV and YFV have even been shown to function as survival factors that can replace IL-3 to maintain the viability of pDCs in vitro [32]. An important impact of the adjuvant aluminium hydroxide on GrB expression could be excluded, as both vaccines containing aluminium hydroxide and vaccines without the adjuvant similarly influenced GrB levels.

So far, no other studies have analysed the influence of prophylactic vaccines on pDC-derived GrB and knowledge about the regulation of GrB in pDCs is scarce. An important cytokine implicated in GrB regulation seems to be IL-3. Although freshly isolated pDCs already produced small amounts of GrB, the expression

markedly increased upon stimulation with IL-3, which goes in line with previous studies [17, 86]. The effect of IL-3 on GrB expression can further be enhanced by IL-10 [86]. Interestingly, synthetic TLR 7 or TLR 9 agonists such as imiquimod for TLR 7 or ODN 2006 for TLR 9 have been discovered to strongly suppress GrB production and secretion by pDCs [17, 86]. Such TLR agonists are frequently used in research to activate immune cells. In case of pDCs, ODNs containing a CpG motive as well as imiquimod have previously been shown to induce both secretion of IFN- α and maturation of pDCs [54, 63]. As the antiviral vaccines used in this study all consist of whole virus particles in either attenuated or inactivated form, the viral RNA might be recognised by pDCs via TLRs and the vaccines might therefore act as suppressors of GrB similarly to the synthetic TLR agonists imiquimod and ODN 2006.

Our group previously reported that T cells coincubated with IL-3-treated pDCs internalise active GrB. T cells in coculture with pDCs expressing very low to no amounts of GrB do not take up GrB, indicating that the GrB within T cells is derived of pDCs [86]. This study showed that T cells cocultured with pDCs matured in the presence of TBEV and IL-3 did not internalise active GrB at early time points despite close pDC - T cell interactions in contrast to T cells incubated with IL3-only-treated pDCs. The observed close interplay between T cells and pDCs in both studies makes it tempting to speculate that IL-3-treated pDCs directly transfer GrB to the T cells, a process that might be inhibited or at least delayed upon vaccine stimulation. However, the precise mechanisms underlying these results definitely deserve future analysis.

The suppression of GrB expression in pDCs by preventive vaccines raises the question of the functional relevance of pDC-derived GrB. The recent findings of our group that GrB-producing pDCs inhibit T cell expansion clearly suggest an immunosuppressive role. The increase of GrB secretion by pDCs upon stimulation with the immunosuppressive cytokine IL-10 [86] and the inhibition of GrB secretion by immunogenic stimuli like vaccines or TLR agonists support this view. Interestingly, Tregs have also been described to reduce T cell proliferation in a similar GrB dependent manner [58]. However, the mechanisms of pDC-derived GrB leading to impaired T cell proliferation are not characterised so far. Cleavage of the TcR ζ -chain [174] or induction of apoptosis as shown for Tregs [58] might be possible explanations to be evaluated. Indeed, one study demonstrated GrB-dependent cytotoxic effects of pDCs [17]. However, the cytotoxicity of pDCs in this

study required high pDC to target cell ratios. Hence, its relevance in vivo may be critically challenged due to the low numbers of circulating pDCs. Irrespective of the mechanism, we hypothesise that unstimulated, immature pDCs are rather tolerogenic and pDC-derived GrB might contribute to suppress autoimmunity under steady state conditions. After vaccination, the decreasing levels of GrB might allow sufficient T cell proliferation to mount an adequate immune response. Clearly, new studies are needed to further investigate this concept.

4.2 Antiviral vaccines modulate phenotype and function of pDCs

Besides the influence of the antiviral vaccines on pDC-derived GrB, the preventive vaccines also modulated IFN- α secretion and surface molecule expression of pDCs. While basically all tested vaccines affected GrB to some extent, only TBEV strongly stimulated IFN- α secretion by pDCs. The secreted amounts of IFN- α are synthesised de novo, as freshly isolated and unstimulated pDCs cultured in IL-3 do not contain preformed intracellular IFN- α [43]. Adjuvant aluminium hydroxide contained in the TBEV did not influence IFN- α secretion at all. Other studies also demonstrated an IFN-inducing effect of vaccines on pDCs [32, 95]. Interestingly, while the single vaccines against measles and rubella failed to mount an INF- α response in the present study, the combination vaccine against measles, mumps and rubella tested by de Vries et al. promoted substantial IFN- α secretion of pDCs [32], possibly due to additive effects or due to the presence of more virus particles. Surprisingly, the yellow fever vaccine which represents one of the most successful and powerful vaccines ever developed leading to long-lasting immunity with persistent neutralising antibodies up to 35 years [135, 136] did not stimulate IFN- α secretion at all, consistent with findings of de Vries's group [32]. In contrast to these results, other studies demonstrated a strong impact of the yellow fever vaccine strain (YF-17D) on the IFN- α response by pDCs [22, 138]. The inconsistent findings might be partially explained by differences in the viral doses used. As the goal of this study was to examine the influence of different vaccines on pDCs, dilutions of the commercially available vaccine were used for stimulation of pDCs. Thus, the exact virus concentrations were not determined. However, the studies demonstrating a positive influence of the yellow fever vaccine strain on pDC-derived IFN- α did not use the vaccine itself but subpassaged the virus from the vaccine in a human colorectal adenocarcinoma cell line and performed the

experiments at a MOI of 1 [22, 138]. Additionally, vaccines produced in chicken embryos can be contaminated e.g. with components of avian retroviruses [80, 169]. De Vries et al. hypothesised that the process of subpassaging the vaccine-derived virus in a human cell line led to different contaminations of the virus in contrast to the vaccine produced in chicken embryos. These diverse contaminants might result in different activation of pDCs which may also explain the contrary results [32].

Corresponding to the results obtained for IFN- α secretion, analysis of pDC surface molecule expression upon stimulation with antiviral vaccines revealed only weak to no modulations in case of IPV, MV and YFV. Only TBEV could be identified as maturation stimulus leading to an upregulation of CD86 by pDCs. Further experiments of our group also demonstrated the induction of CD54 by TBEV [18]. In contrast to these findings, other studies reported considerably stronger effects of prophylactic vaccines on expression of surface molecules for pDCs [32] or conventional DCs [149], indicating that commonly used vaccines represent a strong maturation signal for different dendritic cell subsets. The markedly lower effects seen in the present study may originate from the general supplementation of IL-3 as survival factor. IL-3 itself induces substantial upregulation of a variety of surface molecules including CD54, HLA-DR, CD80 and CD86 in pDCs [62, 89]. With the exception of TBEV, the tested vaccines could not amplify the strong effects of IL-3, thus explaining the moderate results observed in the present study. By contrast, the pronounced effects reported in the study cited above were obtained by stimulating pDCs with vaccines in the absence of IL-3. Compared to freshly isolated and unstimulated pDCs, several vaccines such as TBEV, YFV and others markedly influenced the phenotype of pDCs via induction of costimulatory and antigen-presenting surface molecules to a similar extent as IL-3 [32].

Activating signals including viral stimuli or TLR agonists endow pDCs with an increased capacity to induce strong allogenic T cell proliferation [89, 97]. However, the vaccines in this study only weakly modulated pDC-triggered T cell expansion. A possible explanation for these observations might be strong donor to donor variations and the unknown donors' states of vaccination. An in vivo study in mice demonstrated a critical role of pDCs for an appropriate immune response during primary vaccination against influenza but not for secondary vaccination ("boosting") [95]. Consequently, pDCs from vaccinated donors might show a decreased capacity to stimulate T cell proliferation in MLR experiments compared

to pDCs from unvaccinated individuals. Nevertheless, de Vries et al. described strong allogenic T cell proliferations induced by TBEV-stimulated pDCs compared to IL-3-treated pDCs [32], underpinning an immunogenic influence of the vaccine on pDCs. In contrast to the general supplementation of IL-3 as a survival factor in the present study, de Vries' group used TBEV-treated pDCs without addition of IL-3. As IL-3-induced GrB seems to be an important mechanism by which pDCs suppress T cell expansion [86], and as the vaccines – despite strong decreasing effects on GrB – did not completely abrogate GrB secretion, the remaining GrB in the supernatant might be responsible for the weak proliferative T cell responses observed in the present study. In addition to pDCs, an augmented potential to induce T cell proliferation upon vaccine stimulation has also been reported for conventional DCs [149].

Considered as a whole, these findings indicate an immunogenic effect of preventive vaccines on pDCs (and on other DCs) as the vaccines induce both maturation of pDCs and secretion of proinflammatory cytokines such as IFN- α . Though basically all vaccines tested in this study suppressed GrB to a certain extent, an immunogenic influence of the vaccines on pDC phenotype and cytokine secretion could be mainly demonstrated for TBEV. However, the studies cited above extend this view to several other vaccines and additionally demonstrated an increased capacity of pDCs to induce T cell proliferation upon vaccine stimulation.

4.3 Differences between the pure Edmonston B measles strain and the vaccine strain Schwarz

Some diverging results concerning IFN- α production, GrB secretion and stimulation of T cell proliferation by pDCs upon treatment of the Edmonston B virus strain compared to the measles vaccine (containing the measles strain Schwarz) could be observed in the present study. Both strains are live attenuated viruses with the capability to replicate. While the vaccine failed to induce IFN- α secretion by pDCs, the pure Edmonston B measles virus induced a strong IFN response. The influence of measles virus on pDC-derived IFN- α is also controversially discussed throughout the literature. One study clearly described a suppressive effect on IFN- α production [147], whereas others demonstrated a strong induction of IFN- α in pDCs [37, 38] reflecting the inconsistent results of this study. Certain proteins of the measles virus are known to suppress IFN induction

and signalling [48], e.g. inhibition of TLR-mediated IFN- α induction by the measles virus V protein [130]. These proteins undergo mutations during multiple passaging on different cell cultures leading to attenuated virus strains and consequently to functional differences of measles proteins [7, 8]. The passaging of the two different strains prior to the experiments of the present study might have allowed the occurrence of distinct mutations. Thus, genetic differences among the measles strains may provide an explanation for the incongruent results. Additionally, the Edmonston B strain was propagated in Vero cells, while the measles vaccine is produced in cells of chicken embryos. As discussed above for the YFV, these unequal methods of virus production may lead to different contaminants of the virus derived from the cell cultures that might also contribute to the diverging results.

Besides the unequal IFN- α regulation, the Edmonston B strain also differentially affected GrB. In contrast to the decreased GrB production and secretion upon stimulation with the vaccine containing the Schwarz strain, the pure Edmonston B measles virus showed a tendency to increase GrB secretion. The decreasing levels of intracellular GrB obtained for the pure measles virus might reflect depletion of GrB due to augmented secretion, however, kinetic analyses are needed to further clarify these findings. Further, while vaccine-treated pDCs allowed at least some proliferation of allogenic T cells, pDCs stimulated with the Edmonston B strain significantly reduced T cell expansion, corroborating the inhibitory effect of pDC-derived GrB on T cell expansion. These findings go in line with similar results describing a decreased stimulatory capacity of several other subsets of dendritic cells upon measles virus infection [61, 148, 157] and might reflect the profound immunosuppressive effects which occur during the course of a natural measles virus infection [60]. The answer to the question why the vaccine acts that differently concerning GrB expression remains elusive so far. However, different virus concentrations have to be critically considered as the dilutions of the measles vaccine, where the virus concentration is unknown, are difficult to compare with the MOIs used for experiments with the Edmonston B strain. Generally, further investigations are required to analyse and clarify the varying effects of measles viruses on pDCs reported in this and other studies.

4.4 Contribution of pDCs to vaccination-induced immunity

Most vaccines are thought to confer immunity mainly through the induction of protective antibodies. Antibody titres correlating with protective immunity after vaccination have been proposed for a variety of vaccines against bacterial as well as viral pathogens [132]. Beside this generally accepted view, growing evidence attributes a fundamental function to the innate immune system and in particular to DCs in the context of vaccination, which can orchestrate humoral and cellular adaptive immune responses in multiple ways [137].

In this study, a direct immunogenic influence of the tick-borne encephalitis vaccine on the pDC phenotype was demonstrated. These findings are supported by de Vries et al. and extended to other vaccines [32] suggesting an important function for pDCs in vaccination-induced immune responses. This view is corroborated by an *in vivo* study on mice reporting the crucial involvement of pDCs in mediating immune responses upon vaccination with an inactivated whole-virus vaccine against influenza [95].

Interestingly, the described vaccine-induced effects on pDCs resemble the influence of synthetic TLR agonists like different classes of CpG ODNs that also affect surface molecule expression, cytokine production as well as T cell expansion by pDCs [63, 97, 98]. Of note, the higher order structure of TLR agonists affects the cellular localisation of the molecules and consequently the nature of the cellular response. While large multimeric complexes like CPG ODNs type A are retained in early endosomes and induce a strong IFN- α response in pDCs, single-stranded non-aggregating molecules like CPG ODNs type B localise in late endosomes resulting in lower IFN- α secretion but enhanced maturation of pDCs [63]. This dependence of the pDC response on the higher order structure of the stimuli was also demonstrated for different vaccines [32]. The similarities between TLR agonists and vaccines strongly suggest that the vaccines exert their effects at least partially via TLR activation, most likely via naturally occurring viral TLR agonists e.g. viral DNA or ssRNA contained in the vaccine. Indeed, several studies demonstrated that TLR activation plays an important role for vaccine-mediated immune responses in pDCs and other dendritic cells [32, 95, 138, 149].

The striking differences in particular between the potent TBEV and the other vaccines described in the present study are not clear so far and have to be investigated in future studies. However, aforementioned differences concerning

the production and with it diverse potential contaminants, the higher order structure of the vaccines or unequal contents of viruses and therefore different concentrations of TLR activating stimuli might play a role.

Besides the upregulation of costimulatory molecules and secretion of proinflammatory cytokines, this study additionally described a GrB-inhibiting effect of all the vaccines tested on pDCs. Based on our previous publication indicating an immunosuppressive role for pDC-derived GrB [86], we suggest that the suppression of GrB represents a novel facet of vaccination-induced immunity. Upon engagement of the vaccine by pDCs, the reduced production, secretion and transfer of GrB to T cells might enhance the immune response and thus increase the protective effects of the vaccines. Supporting this concept, pDCs isolated from healthy donors before vaccination produced significantly more GrB than after vaccination [18].

These findings might have implications for future vaccine development. Adjuvants in vaccine preparations used to increase the immunogenicity of the vaccines [15, 133] receive more and more attention. However, few adjuvants are licensed for clinical use and their mechanisms of action are so far only partially understood [29], resulting in a need for the evaluation of new agents. This and other studies discussed above demonstrated an immunogenic influence of several but not all tested vaccines on pDCs. Thus, adjuvants like synthetic agonists for TLR 7 and 9 could be used to selectively stimulate pDC-dependent immune responses and to harness their potent antiviral properties to increase the protection induced by so far rather low immunogenic antiviral vaccines. This view is supported by a study demonstrating that a TLR 9 agonist strongly enhances the immunogenicity of a primarily weak split vaccine against influenza virus in mice [95]. In human trials, the addition of a TLR 9 ligand to hepatitis B or pneumococcal vaccines also increased the immunogenicity of the vaccines [68, 153]. However, as other blood cells e.g. B cells also express TLR 9, the degree of the contribution of pDCs to the increased immunogenicity remains to be evaluated.

In addition to TLR agonists, this study further suggests the potential use of GrB inhibitors as adjuvants to be explored. A combination of TLR agonists resulting in increased expression of costimulatory molecules and cytokine secretion plus the antagonisation of pDC-derived GrB might represent a powerful admixture with respect to an augmented efficiency of future vaccines.

4.5 Vaccine-activated pDCs – a clinical approach

The immunostimulatory effects of preventive vaccines on pDCs might not only be used for the development of improved vaccines but could also be exploited in a wider context. Exemplarily, the dendritic cell-based immunotherapeutic stimulation of an antitumour response shall be discussed here. pDCs have been shown to infiltrate a variety of tumour entities including melanoma [171], head and neck [69], breast [168], ovarian [178], lung cancer [129] and others. In the majority of studies, tumour-associated pDCs predominantly expressed an immature phenotype, produced low amounts of IFN- α in response to TLR agonists, exhibited a decreased T cell stimulatory potential and were able to induce Tregs [69, 129, 171, 173]. Thus, pDCs seem to be influenced by the tumour environment resulting in low immunogenic or tolerogenic properties. Additionally, some neoplasms have been associated with elevated levels of IL-10 [10, 122] with tumour-associated pDCs stimulating T cells to secrete this immunosuppressive cytokine [178]. As IL-10 represents a powerful stimulus for GrB expression by pDCs [86], these findings raise the hypothesis that pDC-derived GrB might further aggravate the immunosuppressive tumour milieu. However, tumour-infiltrating pDCs have not been analysed with respect to GrB production yet. Due to the infiltration of various neoplasms and the hampered immunogenic response within these lesions, pDCs represent an ideal target for dendritic cell-based immunotherapeutic approaches. Reprogramming these cells and restoring their immunogenicity might elicit an immune response directly at the tumour site.

Most studies on DC-based anticancer vaccines use monocyte-derived DC that are differentiated and activated by a mixture of several cytokines. Many results of these trials are not very promising yet which is frequently thought to be a problem of inadequate stimulation of the DCs by the cytokine cocktail [55]. Thus, there is a need for new approaches regarding the development of appropriate stimuli and the use of other DC subsets for example pDCs.

Injection of TLR ligand (CpG)-activated pDCs into mice mounted tumour-specific immune responses associated with the inhibition of tumour growth [107, 109]. However, the low frequency of circulating pDCs strongly limits the feasibility of this approach in human clinical trials. Alternatively, TLR ligands can be delivered directly in vivo to target pDC immune responses in order to circumvent elaborate isolation procedures of pDCs. Peri- or intratumoural application of ODNs reduced

tumour size or even led to tumour remission in mice, although these studies did not assess the direct involvement of pDCs [101, 108]. In phase II trials in humans, subcutaneous or intradermal injection of a TLR 9 agonist resulted in an increased maturation of pDCs in the blood or in the sentinel lymph nodes of melanoma patients, respectively [118, 127]. The activation state of pDCs correlated with higher NK cell infiltration and tumour-specific T cell reactivity in the sentinel lymph nodes [119]. Further, topical treatment of patients with basal cell carcinoma with imiquimod, a TLR 7 agonist, induced tumour regression and was associated with the accumulation of pDCs in the lesions [156]. Whether these observations of pDC activation and accumulation upon TLR agonist application are causally linked with an improved clinical outcome or tumour regression remains to be investigated. Nevertheless, activation of pDCs via TLRs seems to be a promising new immunotherapeutic method to target neoplastic diseases.

The problem with synthetic TLR agonists is that these molecules are not generally licensed for clinical treatment and safety concerns have to be considered. Though first clinical trials in humans indicate no severe side-effects, long-term experiences do not exist so far [96]. The present study in conjunction with de Vries et al.'s work [32] clearly reported an activating effect of preventive vaccines on pDCs in a way reminiscent to synthetic TLR agonists, possibly due to the presence of natural occurring viral TLR agonists in the vaccines. These findings suggest the further evaluation of the vaccines as alternative to synthetic TLR agonists to activate pDCs. The vaccines are cheap, broadly available and generally approved in terms of clinical safety. One in vitro study on conventional DCs demonstrated the generation of highly stimulatory, mature DCs with the ability to present tumour antigens upon stimulation with a cocktail consisting of several vaccines and prostaglandin E2. Of note, these DCs secreted higher amounts of cytokines and induced stronger T cell responses compared to DCs matured with the standard mixture of cytokines. According to the authors, clinical trials are already carried out to test these findings in vivo [149]. Given that pDC-derived GrB plays an immunosuppressive role, the strong inhibitory effects of the vaccines on GrB expression by pDCs reported in the present study might further contribute to an increased immunogenic peritumoural environment. Clearly, these assumptions remain speculation yet and new studies and clinical trials need to be conducted to evaluate the proposed potential of vaccines as activators of pDCs and other DC

subsets in anticancer approaches as well as the role of GrB secretion by pDCs in tumour immunity.

In conclusion, the present study showed a direct immunogenic influence of commonly used antiviral vaccines – in particular of TBEV – on the phenotype of human pDCs. To the best of our knowledge, a suppressive effect of antiviral vaccines on pDC-derived GrB could be demonstrated for the first time. Thus, this study indicates the direct contribution of pDCs to vaccine-induced immune responses *in vitro*. The results provide new insights in the mechanism of action of prophylactic vaccines and suggest the evaluation of GrB inhibitors as novel vaccine adjuvants. Further, the potential of activating pDCs via vaccines as alternative to synthetic TLR agonists might be exploited in immunotherapeutic approaches.

5. Summary

Plasmacytoid dendritic cells (pDCs) are crucial mediators in the context of viral infections and link innate and adaptive immunity. Despite their well-characterised antiviral role, little is known about the contribution of pDCs to vaccine-induced immunity. Beside the immunogenic function, immunoregulatory properties have also been associated with pDCs. A recent study identified the capacity of pDCs to secrete copious amounts of granzyme B (GrB) leading to the suppression of T cell proliferation. However, knowledge about the regulation of pDC-derived GrB is scarce yet. Thus, the aim of this study was to analyse the effects of commonly used antiviral vaccines on pDCs with a special focus set on the regulation of GrB by the vaccines.

For that purpose, pDCs of healthy donors were isolated and purified via magnetic bead isolation and stimulated with different vaccines against polio, measles, yellow fever, rubella and tick-borne encephalitis. After 24 hours of stimulation, GrB production and secretion were measured via flow cytometry and GrB enzyme-linked immunosorbent assay (ELISA), respectively. In addition to GrB, the secretion of the proinflammatory cytokine interferon (IFN)- α , a hallmark of activated pDCs, was quantified via ELISA. Furthermore, the phenotype of pDCs was characterised by analysis of surface molecule expression. The immunogenic potential of pDCs to induce T cell expansion upon stimulation with vaccines was evaluated in mixed lymphocyte reaction experiments. Finally, transfer of GrB from pDC to T cells was investigated via spinning disc confocal microscopy.

GrB production and secretion decreased upon stimulation with all the tested vaccines, however to different extents and with polio, measles and tick-borne encephalitis vaccine (TBEV) being the most potent suppressors of GrB expression. Interestingly, pDCs stimulated with interleukin (IL)-3 and TBEV did not transfer GrB to T cells as opposed to pDCs treated with IL-3 only. In contrast to the general influence of the vaccines on pDC-derived GrB, only TBEV induced a substantial IFN- α response in pDCs. Consistent with these findings, surface molecule expression was only weakly modulated by the vaccines except TBEV which strongly increased the expression of cluster of differentiation (CD)86, a costimulatory molecule. Despite strong secretion of IFN- α and upregulation of CD86 in case of TBEV, the vaccines generally failed to significantly enhance the

potential of pDCs to induce allogenic T cell proliferation. However, substantial variations between different donors of pDCs and T cells could be observed.

The immunogenic influence of commonly used antiviral vaccines on pDCs – described in the present study especially for TBEV – is supported by other studies which have extended this view to further vaccines and conventional dendritic cells (DCs).

Interestingly, the response of pDCs upon vaccine stimulation resembled described effects of synthetic toll-like receptor (TLR) agonists on pDCs. In fact, several studies demonstrated the involvement of TLR signalling in vaccine-mediated immune responses. Hence, the addition of TLR agonists to vaccines might be a valuable tool to increase the efficiency of so far less potent vaccines by harnessing the immunostimulatory properties of pDCs. Besides, immunogenic vaccines might serve as clinically available TLR agonists to activate pDCs and other DCs in terms of immunotherapeutic approaches, e.g. anticancer vaccines.

In conclusion, the findings of the present work in conjunction with other studies suggest a direct contribution of pDCs to immune responses upon vaccination. The general inhibitory effect of the vaccines on pDC-derived, immunosuppressive GrB reported in the present study indicates a novel mechanism of how vaccines affect the immune system and proposes the evaluation of GrB inhibitors as adjuvants for future vaccines.

6. References

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