

The role of G-quadruplexes in myeloid dendritic cells

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Abbreviations

°C	degrees Celcius
µg	microgram
µl	microlitre
µM	micromolar
allo-HCT	allogeneic hematopoietic cell transplantation
aGvHD	acute GvHD
AP-1	activator protein 1
APC	antigen-presenting cell
ATP	adenosine triphosphate
Batf3	Basic Leucine Zipper ATF-like Transcription Factor 3
BCL	B-cell lymphoma
BM	bone marrow
BMDCs	bone marrow-derived dendritic cells
BSA	bovine serum albumin
CCR	C-C chemokine receptor
cCRE	candidate cis-regulatory element
CD	cluster of differentiation
cDC	conventional dendritic cell
CDK	cyclin-dependent kinase
CDP	common DC progenitor
CFSE	carboxyfluorescein succinimidyl ester
cGvHD	chronic GvHD
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor

CMTM6	CKLF-like MARVEL transmembrane domain-containing protein 6
CNBP	cellular nucleic acid-binding protein
CO ₂	carbon dioxide
CpG ODN	CpG oligodeoxynucleotide
CRC	colorectal cancer
crRNA	CRISPR RNA
CSN5	COP9 signalosome 5
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DAMP	damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DDR	DNA damage response
DEG	differential expressed gene
DIG	digoxigenin
dLN	draining lymph node
DMEM	Dulbecco's Modified Eagle Medium
DSB	double-strand break
DSCs	decidua stromal cells
EAE	experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
FDA	Food and Drug Administration
FLT3L	FMS-like tyrosine kinase 3 ligand

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G3BP1	GTPase-activating protein-binding protein 1
GAS	gamma-activated sequence
GM-CSF	granulocyte-macrophage colony-stimulating factor
G4	G-quadruplex
gMFI	geometric mean fluorescent intensity
GO	gene ontology
gRNA	guide RNA
GSK3 β	glycogen synthase kinase 3 β
GvHD	Graft-vs-Host-Disease
GvL	Graft-vs-leukaemia
Gy	Gray (unit)
h	hour
H3c10	H3 clustered histone 10
HDAC	histone deacetylase
HIF1 α	hypoxia-inducible factor 1 alpha
HLA	human leukocyte antigen
HMGB1	high-mobility group box 1
HR	homologous recombination
HRE	hypoxia response element
HSC	hematopoietic stem cell
HU	hydroxyurea
iDC	inflammatory DC
IDO	indoleamine-2,3-dioxygenase
Ig	immunoglobulin
IF	immunofluorescence
Ifit	interferon-induced protein with tetratricopeptide

	repeats
IFN	interferon
IL	interleukin
IMDM	Iscove's Modified Dulbeccos Medium
i.p.	intraperitoneal
IRF	interferon regulatory factor
ISG	interferon-stimulated gen
i.t.	intratumourally
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
i.v.	intravenously
JAK	Janus kinase
KD	knock down
KLF	Krüppel-like factor
KO	knock out
LAG-3	lymphocyte-activation gene 3
LN	lymph node
LPS	lipopolysaccharide
mA	milliampere
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MD	molecular dynamics
MDA-5	melanoma differentiation-associated protein 5
MDP	monocyte-macrophage DC progenitor
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex

Min	minutes
miRNA	microRNA
mL	millilitre
moDCs	monocyte-derived dendritic cells
mRNA	messenger RNA
MSC	mesenchymal stromal cells
mTOR	mammalian target of rapamycin
Mx	Mx dynamin like GTPase
NFIL3	Nuclear Factor, Interleukin 3 Regulated
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NGS	next generation sequencing
NHEJ	nonhomologous DNA end joining
NK	natural killer cell
NLRC4	Nod-like receptor (NLR) family CARD domain containing 4
NLRP3	NLR family pyrin domain containing 3
NOD	nucleotide-binding oligomerisation domain
NOD	non-obese diabetic
Notch	Neurogenic locus notch homolog protein
NSCLC	non-small-cell lung cancer
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PD-1	Programmed death 1
PDAC	pancreatic ductal adenocarcinoma
pDC	plasmacytoid dendritic cell
PD-L1	Programmed death-ligand 1
PDS	pyridostatin
Pen/Strep	Penicillin/Streptomycin
PFA	paraformaldehyde
pg	picogram
PGE2	Prostaglandin E2
PI3K	phosphoinositide 3-kinase
Poly I:C	polyinosinic:polycytidylic acid
PQS	putative/predicted G-quadruplex sequence
PRR	pattern recognition receptor
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
RCC	renal cell carcinoma
RF	restriction free
RIF1	Rap1-interacting factor 1
RIG-I	retinoic acid-inducible gene I
RPMI	Roswell Park Memorial Institute
RSAD2	Radical S-Adenosyl Methionine Domain Containing 2
RT	room temperature
RTP4	receptor transporter protein 4
s.c.	subcutaneous

SDS	sodium dodecyl sulfate
SG	stress granule
SLE	systemic lupus erythematosus
SNV	single nucleotide variant
Socs	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TAA	tumour-associated antigen
TAM	tumour-associated macrophage
TBE	Tris/Borate/EDTA
TBI	total body irradiation
TBS	tris-buffered saline
TBST	TBS with Tween20
TCD	T cell depletion
TCR	T cell receptor
tDCs	tolerogenic DCs
tdLN	tumour-draining lymph nodes
TF	transcription factor
TGF	transforming growth factor
T _h	T helper cell
ThT	Thioflavin T
TIA1	T cell-restricted intracellular antigen-1
TIM-3	T cell immunoglobulin and mucin domain
TLE	transducin-like encancer
TLR	toll-like receptor
TME	tumour microenvironment
TNF	tumour-necrosis factor

TPP1	tripeptidyl-peptidase 1
tracrRNA	transactivating crRNA
T _{reg}	regulatory T cell
TRF	telomeric repeat-binding factor
TSS	transcription starting site
USP	ubiquitin-specific peptidase
UTR	untranslated region
V	volt
VEGF	vascular endothelial growth factor
WB	western blot
w/o	without
ZBP1	Z-DNA binding protein 1

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Abstract

DNA can fold into different secondary structures such as G-quadruplexes (G4s). G4s are highly stable and can form in specific guanine-rich regions within the genome both *in vitro* and *in vivo*. Due to their conserved localisation, DNA G4s are suggested to affect different cellular processes, such as DNA replication, gene expression and telomere maintenance. Their formation is cell type specific and dynamically regulated. G4 structures have already been investigated in several cell types, including stem cells and cancer cells, but little is known about G4s in immune cells such as dendritic cells (DCs). DCs are antigen-presenting cells that link innate and adaptive immunity by inducing an antigen-specific immune response. They play a crucial role, particularly in tumour immunity by inducing anti-tumour-specific T cell responses. Current mechanisms of immunotherapeutic strategies focus on boosting immune responses towards cancer cells e.g. through adoptive cell transfer, DC-based vaccines or stimulatory cytokines. Hence, a better understanding of G4 formation and their relevance for DC function could assist specific targeting and regulation of DC function on the genomic level to induce an effective anti-tumour immune response.

In this study, I characterised the molecular effect of G4 stabilisation on DC phenotype and function using the G4 ligand pyridostatin (PDS). Stabilisation of G4s in bone marrow-derived dendritic cells (BMDCs) induces DC maturation and activation by elevating the surface expression of co-stimulatory molecules (CD40, CD80 and CD86), MHC molecules and the enhancement of DC migration. Transcriptomic analysis revealed that G4 stabilisation leads to transcriptional reprogramming in DCs, involving the upregulation of genes associated with DC maturation.

In addition, I investigated the potential of G4 stabilisation in DCs as an immunotherapeutic target in B16OVA melanoma and Graft-vs-Host-Disease mouse models. I could show that intratumoral injection of PDS enhanced DC activation within the tumour. In line with this, type I conventional DCs (cDC1s) almost completely disappeared from tumours following PDS treatment. Besides its effectivity in B16OVA melanoma, regular intraperitoneal injections of PDS

enhanced the survival and attenuated GvHD-specific symptoms like weight loss, fur loss and skin rash in GvHD-bearing mice, thereby reducing the severity of acute GvHD.

In this study, I further investigated the molecular mechanisms underlying G4-induced cellular changes in DCs by the immune-inhibitory molecule PD-L1. Genome-wide mapping discovered a G4 motif in the PD-L1 gene promoter and G4 stabilisation with PDS resulted in elevated PD-L1 expression. Using the CRISPR/Cas9 system, I could show that the effect of G4 stabilisation on PD-L1 expression is dependent on the transcription factor STAT3. The role of STAT3 in G4-mediated PD-L1 expression was further strengthened by the binding of STAT3 to the G4 structure in the PD-L1 promoter.

In summary, this thesis contributes to a better understanding of G4 formation and its role in cellular pathways in DCs by providing insights into how G4 stabilisation affects DC phenotype and function and exploring the underlying mechanisms of G4-mediated cellular changes. These findings will broaden the knowledge of how DCs can be regulated on the genomic level to improve the induction of T cell-mediated immune responses, a requirement for most therapeutic approaches.

Declaration

This is to certify that

- (i) the thesis comprises only my original work towards the PhD except where indicated in the preface,
- (ii) due acknowledgement has been made in the text to all other material used; and
- (iii) This thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Preface

This work was conducted in the laboratories of Prof. Dr. Katrin Paeschke and Prof. Dr. med. Annkristin Heine at the University of Bonn and in the laboratories of Prof. Dr. Justine D. Minter at the University of Melbourne. The work was funded by the GRK2168 (Bonn-Melbourne Graduate School).

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Chapter 3: Next generation Sequencing was conducted by the NGS Core Facility (University of Bonn). Proportion of original work by the author: 98 %

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List of publications

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1. Introduction

1.1 Dendritic cells

In 1868, Dendritic cells (DCs) were first described by Paul Langerhans as cutaneous nerve cells in human skin (Langerhans, 1868). However, the term “dendritic cell” was shaped by Steinman and Cohn, who discovered the function of DCs in 1973 (Steinman and Cohn, 1973). DCs are professional antigen-presenting cells (APCs) that link innate and adaptive immunity by sensing invading pathogens or damaged cells followed by initiating an antigen-specific immune response to eradicate them (Steinman, 2006). Besides their function in antigen-specific adaptive immunity, DCs play a critical role in the induction of tolerance against self-antigens and the regulation of immune homeostasis (Waisman et al., 2017). Their enormous significance is evident in patients suffering from diseases caused by impaired/altered DC functions, such as common allergies and autoimmune diseases like systemic lupus erythematosus (SLE) and inflammatory bowel diseases as well as the rare blastic plasmacytoid dendritic cell neoplasm (Baumgart et al., 2009; Blomberg et al., 2001; Owczarczyk-Saczonek et al., 2018).

1.1.1 Mouse dendritic cell classification

DCs can be found in various lymphoid organs, such as the thymus, spleen, and lymph nodes, but also in non-lymphoid tissues, including skin, gut, lung, and liver as well as in peripheral blood (Balan et al., 2019). DCs are a heterogeneous population that arises from hematopoietic stem cells in a multi-step process (Figure 1). Mouse DCs can be broadly classified as steady-state DCs and inflammatory DCs (iDCs). Steady-state DCs derive from a common DC progenitor (CDP) in a FMS-like tyrosine kinase 3 ligand (FLT3L)-dependent manner (Maraskovsky et al., 1996). iDCs generally differentiate from LyC6⁺ monocyte precursors in a macrophage colony-stimulating factor (M-CSF)-dependent manner during inflammation (Segura and Amigorena, 2013). Steady-

state DCs can be divided into two subtypes, conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs).

pDCs originate from a common lymphoid progenitor (CLP) or a CDP and have the capacity to produce high amounts of Type I interferon (IFN) (Swiecki and Colonna, 2015). In addition, mouse pDCs play an important role in inducing tolerance and providing protection against various pathogens and viral infections (Bonaccorsi et al., 2010; Dasgupta et al., 2014; Tezuka et al., 2011). The differentiation from a common DC precursor in the bone marrow (BM) into a pDC is mainly regulated by the transcription factor (TF) E2.2 but is additionally dependent on the interaction between several other regulatory proteins, such as BCL11A, Spi-B, Interferon Regulatory Factor (IRF) 8, Zeb2, Ikaros and Runx2 (Reizis, 2019). Mouse pDCs can be identified by the expression profile of CD11c, major histocompatibility complex (MHC) II, CD45R (B220), CD317 (BST2) and Siglec H (Swiecki and Colonna, 2015). Expression of E2.2 is counterbalanced by an inhibitor of DNA binding 2 (ID2), thus inhibition of E2.2 in CDP leads to the differentiation into conventional DCs (Bagadia et al., 2019).

Conventional DCs are the main antigen-presenting DCs and can further be classified into conventional type I DC (cDC1) and conventional type II DC (cDC2). cDC precursors (pre-cDCs) leave the BM to complete their terminal development in peripheral organs (Diao et al., 2006; Naik et al., 2005). Differentiation into cDC1 is dependent on the TFs IRF8, PU.1, NFIL3 (Nuclear Factor, Interleukin 3 Regulated), ID2 and Basic Leucine Zipper ATF-like Transcription Factor 3 (Batf3) (Chandra et al., 2017; Chopin et al., 2019; Grajales-Reyes et al., 2015; Kashiwada et al., 2011; Sichien et al., 2016). In contrast, cDC2 development is mainly regulated by IRF4 (Schlitzer et al., 2013). Mouse cDC1s and cDC2s can be distinguished phenotypically based on a specific surface molecule profile (Shortman and Heath, 2010). cDC1s typically express CD11c, MHC II, XCR1, Clec9A and CADM1. In addition, the expression of CD8 α serves as a marker for lymphoid-resident cDC1s, while CD103 is generally expressed by the migratory subset (Bedoui et al., 2009). cDC2s are distinguished by the expression of CD11c, MHC II, CD11b and CD172 (SIRP1 α). cDC2s represent a heterogeneous

population in the spleen and intestine and can further be subdivided into two subsets based on the expression of Clec12A and ESAM. Development of Clec12A^{low}ESAM^{high} cDC2s (cDC2A) was described to be dependent on Neurogenic locus notch homolog protein 2 (Notch2) signalling, while the development of Clec12A^{high}ESAM^{low} cDC2s (cDC2B) was found to be dependent on the TF Kruppel-like factor 4 (KLF4) (Lewis et al., 2011; Tussiwand et al., 2015).

The cDC subsets also differ in the pattern recognition receptors (PRRs) that they express on the cell surface or intracellularly to recognise different pathogens. cDC1s sense pathogen-associated molecular patterns (PAMPs) through Toll-like receptor (TLR)2, 3, 4, 9, 11, 12 and 13 (Edwards et al., 2003; Luber et al., 2010). Importantly, cDC1s do not express TLR7 and poorly express cytosolic PRRs such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5) and different inflammasome components, including NRLC4 (Nod-like receptor (NLR) family CARD domain containing 4), NLRP3 (NLR family pyrin domain containing 3) and Z-DNA binding protein 1 (ZBP1) (Luber et al., 2010). cDC2s express a broad range of TLRs, except for TLR3 and TLR12. Importantly, TLR expression and response to specific TLR ligands is cDC2 subset-specific. In contrast to cDC1s, cDC2s express a variety of cytosolic nucleic acid sensors (RIG-I, MDA-5, ZBP1) and Nod-like receptors (NOD1, NLRX1) that enable them to detect various pathogens within the cytoplasm (Edwards et al., 2003; Luber et al., 2010).

The two cDC subsets do not only vary phenotypically but also functionally. Their specific function within the immune system will be further described in the following section.

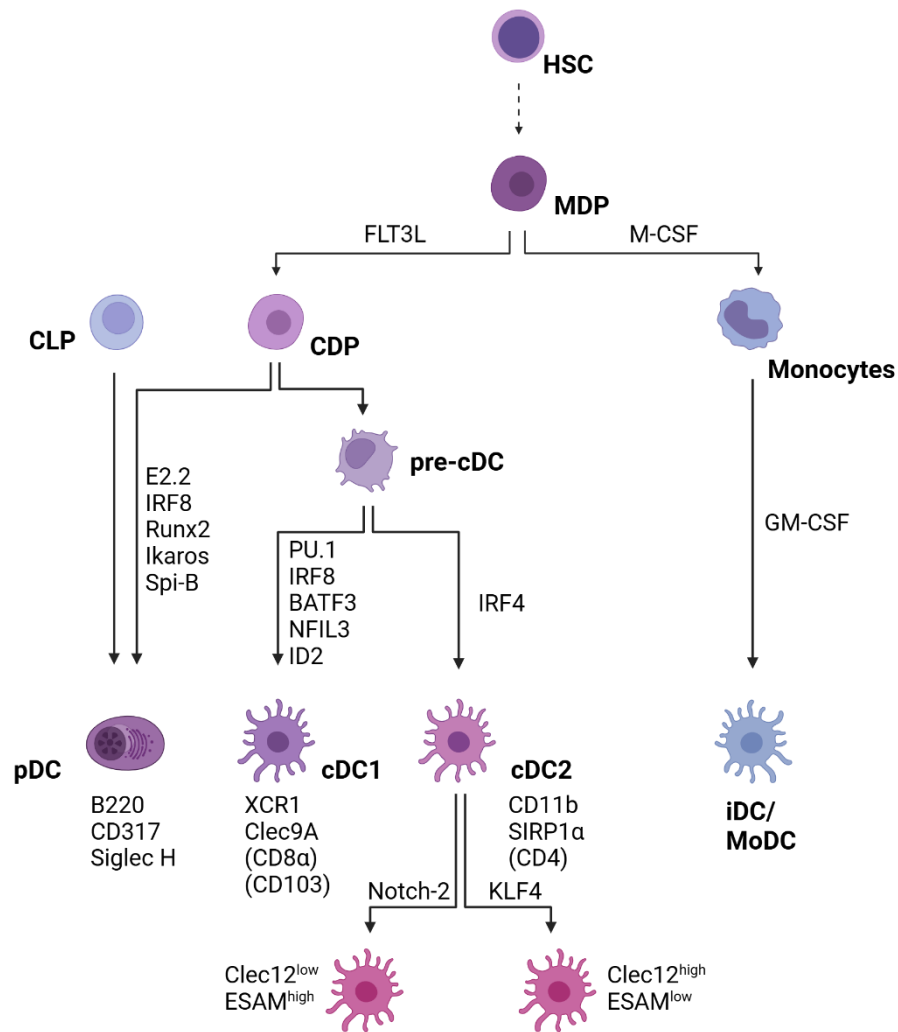


Figure 1.1 | Dendritic cell ontogeny. Dendritic cells originate from hematopoietic stem cells in a multi-step process. Inflammatory DCs (iDCs) differentiate from monocytes during inflammation. Steady-state DCs generally originate from a common DC progenitor (CDP) with the potential to give rise to different DC subsets, including conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can further be divided into type I cDCs and type II cDCs. HSC: hematopoietic stem cell, MDP: monocyte-macrophage DC progenitor, CDP: common DC progenitor, CLP: common lymphoid progenitor, cDC: conventional DC, pDC: plasmacytoid DC, iDC: inflammatory DC, MoDC: monocyte-derived DC, FLT3L: FMS-like tyrosine kinase 3 ligand, GM-CSF: granulocyte-macrophage colony-stimulating factor, IRF: interferon-regulatory factor, KLF4: Krüppel-like factor 4, NFIL3: nuclear factor interleukin 3, BATF3: basic leucine zipper ATF-like transcription factor 3. Figure created with Biorender.

1.1.2. The function and role of dendritic cells in immunity

The main function of DCs as antigen-presenting cells is to recognise danger signals and to phagocytose, process and present them as antigenic peptides on MHC I and MHC II molecules on their surface to T cells to induce an adaptive immune response. Pre-DCs migrate from the BM into peripheral tissues, where they develop into immature DCs, which act as sentinels, monitoring the extracellular environment for foreign antigens (Banchereau et al., 2000). DCs recognise PAMPs and damage-associated molecular patterns (DAMPs) by specific PRRs on their surface, including TLRs and C-type lectin receptors or cytoplasmic PRRs such as RIG-I or MDA-5 (Diebold, 2009). Recognition of antigens by PRRs leads to DC activation (further described in section 1.1.3), resulting in antigen uptake and processing into peptides, which will further be loaded onto MHC molecules. During this process, DCs migrate to the draining lymph nodes (dLNs), where they present the antigenic peptides to naïve CD4⁺ and CD8⁺ T cells via MHC I and MHC II molecules, which leads to T cell activation and the induction of adaptive immune responses (Banchereau et al., 2000). T cell activation by dendritic cells requires in total three signals: stimulation via the recognition of the antigenic peptide/MHC complexes by the T cell receptor (TCR) (Signal 1), co-stimulation provided by the triggering of CD28 on T cells via CD80 (B7.1) and CD86 (B7.2) expressed on DCs (Signal 2), required to sustain T cell activation, and T cell polarization via DC secreted inflammatory cytokines (Signal 3). Peptides generated from endogenous antigens (self-antigens) are generally presented on MHC I molecules to CD8⁺ T cells, whereas exogenous peptides are presented on MHC II to CD4⁺ T cells (Mantegazza et al., 2013). However, some DCs, including CD8α⁺ DCs, can cross-present exogenous antigens on MHC I to CD8⁺ T cells. This process allows DCs to trigger responses against intracellular antigens derived from other cell types (non-DCs) (Kurts et al., 2010). Upon DC interaction, CD8⁺ T cells induce a cytotoxic immune response by releasing cytotoxic granules to eliminate cancer, pathogenic and damaged cells (Halle et al., 2017). Interaction of DCs with naïve CD4⁺ T cells leads to their differentiation into various T helper (T_h) cell types, such as T_h1, T_h2 and T_h17, which is dependent on certain factors, including type of presented antigen (bacterial, viral)

and types of immunostimulatory cytokines expressed by DCs (Interleukin (IL)-12, IL-33, IL-4, IL-5, IL-6, IL-17, IL-23). While T_H1 responses are directed against intracellular pathogens, T_H2 responses are important for the clearance of extracellular parasites and T_H17 responses control infections by extracellular bacteria and fungi at barrier surfaces (Lewis et al., 2011; Mashayekhi et al., 2011; Satpathy et al., 2013; Tussiwand et al., 2015). T_H cells primed by DCs can interact with B cells and stimulate antigen-specific antibody production (Sornasse et al., 1992). Besides T cells, it has been shown that DCs in lymphoid tissues can also interact with and prime natural killer (NK) cells and B cells (Lucas et al., 2007; Wykes and Macpherson, 2000).

In addition to the induction of antigen-specific immune responses, DCs are also critical for the induction of central and peripheral tolerance against self-antigens and the regulation of immune homeostasis (Waisman et al., 2017). In the thymus, DCs participate in the control of $CD4^+$ T cells that react to self- or auto-reactive antigens. Self-antigen presentation by thymic DCs can either result in the elimination of auto-reactive T cells (negative selection of thymocytes) or the generation of thymus-derived regulatory T cells (T_{regs}) (central tolerance) (Hubert et al., 2011; Lei et al., 2011). Under steady-state conditions, DCs continuously present self- or harmless foreign antigens to T cells in the absence of infection and inflammation, maintaining immune homeostasis and promoting peripheral tolerance (Steinman et al., 2003; Steinman and Nussenzweig, 2002). Growing evidence suggests that DCs regulate peripheral tolerance by inhibiting T cell responses via upregulation of inhibitory molecules or by inducing T cell anergy, T cell depletion or T_{reg} cell development (Horton et al., 2017). However, disruption of self-tolerance through pro-inflammatory environmental conditions or genetic alterations induce DC maturation and their production of various inflammatory cytokines to initiate differentiation of auto-reactive T and B cells, resulting in autoimmunity (SLE, psoriasis, Type-I diabetes) (Ganguly et al., 2013).

Conventional DCs are the main antigen-presenting cells required for the induction of an appropriate adaptive immune response. The two cDC subsets described above reveal different specificities in their function to induce T cell immunity.

cDC1s have the specific capacity to cross-present exogenous and aberrant self-antigens on MHC I to activate naïve CD8⁺ T cells for the elimination of cancer cells, damaged cells or pathogenic cells (Bedoui et al., 2009; Den Haan et al., 2000; Ghislat et al., 2021; Hildner et al., 2008; Svensson-Arvelund et al., 2022). Moreover, cDC1s are the main producers of IL-12, which is required for CD8⁺ T cell activation, and play a major role in licensing CD4⁺ T cells for CD8⁺ T cell activation (T_h1 immune response) (Ashour et al., 2020; Ferris et al., 2020). Compared to cDC1s, cDC2s are more efficient in presenting antigens via MHC II to CD4⁺ T cells (Dudziak et al., 2007; Kamphorst et al., 2010). This results in CD4⁺ T cells differentiating into T helper cells or T_{reg}s. cDC2s play a key role in the induction of T_h2 and T_h17 immune responses to eliminate extracellular pathogens and parasites (Kumamoto et al., 2013; Mount et al., 2008; Schlitzer et al., 2013). The direction of these distinct responses by cDC2s is highlighted by their heterogeneous population. Furthermore, cDC2s are important for autoimmunity and tolerance by contributing to T_{reg} development and inhibiting self-reactive CD4⁺ T cells in the thymus (Proietto et al., 2008).

1.1.3 Features of dendritic cell maturation and activation

Dendritic cell maturation is a continuous process that is initiated in the peripheral tissues upon antigen capture or due to inflammatory cytokines secreted by surrounding immune cells and is completed during DC-T cell interaction (Banchereau et al., 2000) (Figure 2). Precursor DCs (Pre-DCs) extravasate from the BM into lymphoid organs and peripheral tissues such as skin and most solid organs, where they give rise to immature DCs. At steady state, immature DCs express high levels of CD11c, intermediate levels of MHC II, low levels of co-stimulatory molecules such as CD40, CD80, and CD86 and lack pro-inflammatory cytokine production (IL-12, IL-1 β and IL-6) (Hawiger et al., 2001). Immature DCs patrol in tissues to recognise PAMPs and DAMPs via PRRs on their surface such as TLRs and C-type lectins or intracellular sensors like RIG-I and MDA-5 (Diebold, 2009). Recognition of “danger signals” in response to invading pathogens or damaged self-antigens initiates DC activation. Upon signal

recognition, antigen uptake can occur through different mechanisms such as macropinocytosis, receptor-mediated endocytosis or phagocytosis (Albert et al., 1998; Fanger et al., 1996; Sallusto et al., 1995). DC maturation is a highly regulated process associated with several events such as loss of antigen-capture activity, increased expression of MHC molecules, upregulation of co-stimulatory molecules (e.g. CD40, CD80 and CD86) and increased production of immunomodulatory cytokines (Banchereau et al., 2000; Reis E Sousa, 2006). In addition, DCs are also reported to undergo morphological changes during maturation, including loss of adhesive structures, cytoskeleton reorganisation and acquisition of high cellular motility (Winzler et al., 1997). During the maturation process, DCs upregulate the C-C chemokine receptor 7 (CCR7), which allows the cells to exit the peripheral tissues and migrate to the lymph nodes via chemokine gradient to present antigenic peptides on MHC I and II molecules to CD4⁺ and CD8⁺ T cells, inducing T cell priming (Sozzani et al., 1998; Dieu et al., 1998; Sallusto et al., 1998; Gunn et al., 1999; Förster et al., 1999).

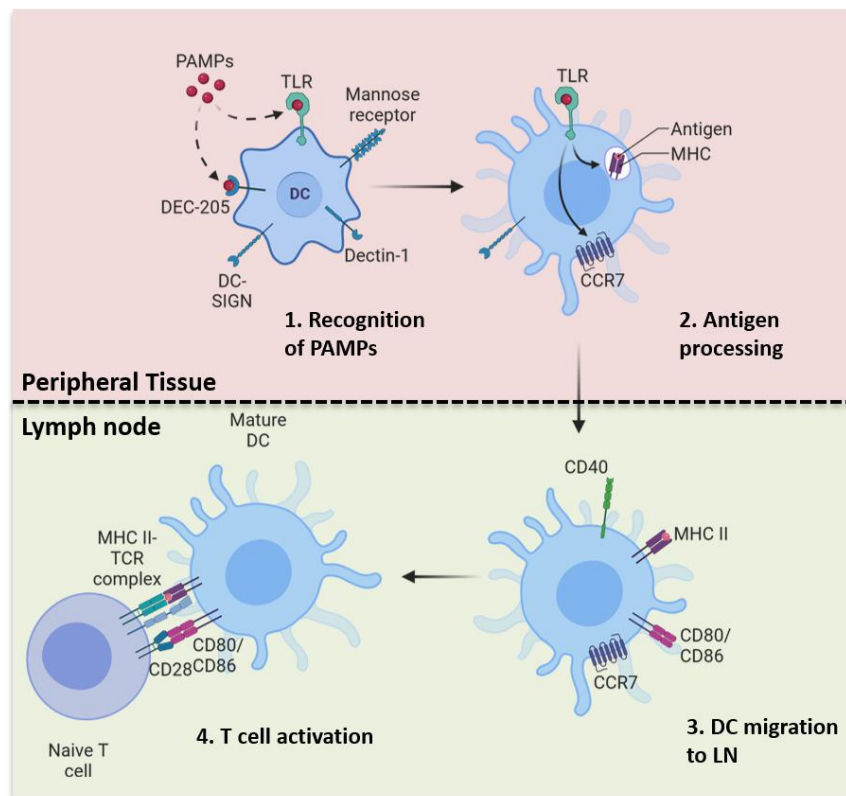


Figure 1.2 | The dendritic cell maturation process. Immature dendritic cells (DCs) patrol in tissues to recognise pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) to become activated (1). Upon antigen recognition, the antigen is taken up, processed and loaded onto major histocompatibility complex (MHC) molecules (2). DC maturation is associated with several events, including increased expression of peptide-loaded MHC molecules, upregulation of the co-stimulatory molecules CD40, CD80 and CD86 as well as upregulation of the C-C chemokine receptor CCR7, which allows the cells to migrate to the lymph nodes (3). In the lymph nodes, DCs present the antigenic peptides on MHC molecules to naïve T cells, whose priming is supported by the interaction of CD28 with CD80 and CD86 (4). TLR: toll-like receptor, MHC: major histocompatibility complex. Figure created with Biorender.

1.2 The role of dendritic cells in tumour immunology

DCs play a critical role in various clinical implications including anti-viral immunity, anti-tumour immunity and autoimmune diseases. Especially in the field of tumour immunology and transplantation, DCs have gained great attention as a potential target for therapeutic approaches.

1.2.1 Cancer

Cancer is the second leading cause of mortality worldwide (World Health Organization, 2024: Cancer). It represents a group of diseases characterised by an uncontrolled proliferation of cells. This abnormal proliferation is caused by a series of mutations in so-called proto-oncogenes and tumour suppressor genes, which are often involved in the cell cycle, controlling cell growth, cell division and apoptosis (Weinberg, 2007). Cancer development and progression are highly affected by interactions of cancer cells with their microenvironment. The tumour microenvironment (TME) is a dynamic and complex milieu that encompasses cancer surrounding and infiltrating cells, signalling molecules, extracellular matrix components and the vascular system (Balkwill et al., 2012). In addition to malignant cells, the TME is composed of various immune cells, such as macrophages, dendritic cells, neutrophils, mast cells, T cells, B cells, NK cells and myeloid-derived suppressor cells (MDSC) and non-immune cells, like fibroblasts, pericytes, vascular and lymphatic endothelial cells. Based on its composition, including immunosuppressive cell populations and the metabolic environment, the TME inhibits the effector functions of immune cells to combat the tumour (Labani-Motlagh et al., 2020).

1.2.1.1 The role and function of dendritic cells in the TME

DCs play a key role in mediating anti-tumour immunity. They are recruited into the TME by chemokines such as CCL4/CCL5/XCL1 secreted by tumour and NK cells and recognise DAMPs released by dying tumour cells (Barry et al., 2018). DCs transport tumour-associated antigens (TAAs) to secondary lymphoid organs, where they cross-present them via MHC I to CD8⁺ T cells, inducing an anti-tumour immune response (Roberts et al., 2016). However, DC function can be altered by several pathways in the TME, leading to tumour immune escape (Jhunjunwala et al., 2021). Immunosuppressive mediators such as Prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), hypoxia and lactate produced by tumour and stromal cells can block activation, maturation and differentiation of

DCs in the TME (Elia et al., 2008; Gabrilovich et al., 1996; Gottfried et al., 2006; Hangai et al., 2016; Suthen et al., 2022). Besides MDSCs and tumour-associated macrophages (TAMs), T_{regs} are the major immunosuppressive population in the TME that induce DC dysfunction. One mechanism of T_{reg}-mediated suppression involves the constitutive expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) on T_{regs} that competes with CD28 on T cells for the co-stimulatory molecules CD80 and CD86 on the DC surface. CTLA-4 depletes CD80/86 on DCs via trans-endocytosis, reducing CD28 signalling and T cell activation (Qureshi et al., 2011). Furthermore, CTLA-4 interaction with CD80 or CD86 induces the production of indoleamine-2,3-dioxygenase (IDO) in DCs, resulting in the suppression of effector T cell activation (Fallarino et al., 2003). Further interactions with co-stimulatory molecules to interfere with the stimulatory signalling between DCs and effector T cells (e.g. lymphocyte-activation gene (LAG)-3/MHC II) and direct induction of death through mutual contact with DCs are additional capabilities of T_{regs} to prevent DC-mediated tumour immunity (Liang et al., 2008; Boissonnas et al., 2010).

Due to the several factors impeding DC function in the TME, a possible therapeutic strategy to overcome DC dysfunction and enhance anti-tumour immunity would be to target immune tolerance pathways (e.g. programmed-death 1 (PD-1)/programmed-death ligand 1 (PD-L1) blockade, T cell immunoglobulin and mucin domain (TIM)-3 blockade, CTLA-4 blockade on T_{regs}) (Arce Vargas et al., 2018; De Mingo Pulido et al., 2018; Hollebecque et al., 2021; Oh et al., 2020; Peng et al., 2020; Simpson et al., 2013) or soluble factors (VEGF, PGE2, IDO) in the TME (Kjeldsen et al., 2021; Pelly et al., 2021; Tada et al., 2018; Tang et al., 2021).

As key activators of immune responses, DCs themselves provide a promising target for anti-tumour therapies. Primary strategies based on DCs include the administration of immune enhancers, such as FLT3L, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CpG ODN (TLR9 ligand) that promote the generation and activation of DCs (Bhardwaj et al., 2020; Oosterling et al., 2006; Sluijter et al., 2015). Another approach describes the fusion of tumour

antigens to targeted antibodies against DEC205 or Clec9a to support the transport of antigens to DCs and thus enhance DC-induced immune responses (Bhardwaj et al., 2020; Dhodapkar et al., 2014; Masterman et al., 2020). The most popular strategies to stimulate an effective anti-tumour immune response are DC-based cancer vaccines. The working principle involves the injection of *ex vivo* stimulated DCs loaded with TAAs (whole tumour lysate) or DCs transfected with messenger RNA (mRNA) encoding cancer antigens into the patient's body to trigger an anti-tumour immune response (Gu et al., 2020). Several DC vaccines have been tested in multiple clinical trials. In these trials, DC vaccines have been shown to be a powerful anti-tumour immunotherapeutic strategy by activating an immune response able to eliminate cancer cells and producing long-lasting immunity in various cancer types, including glioblastoma, acute myeloid leukaemia, colorectal cancer, non-small cell lung cancer and melanoma (Batich et al., 2017; Caballero-Baños et al., 2016; Khoury et al., 2017; Kimura et al., 2018; Wilgenhof et al., 2016).

The importance of DCs in tumour immunity and the positive effect of DCs as a therapeutic target highlight the need for a better understanding of DC biology to facilitate the development of novel DC-targeted therapeutic approaches to promote current anti-tumour immunotherapeutic strategies.

1.2.2 Acute Graft-vs-Host-Disease

Allogeneic haematopoietic cell transplantation (allo-HCT) is an effective therapy for a variety of malignant and non-malignant haematological diseases (Copelan, 2006). Patients receive haematopoietic stem and progenitor cells from a human leukocyte antigen (HLA)-matched donor to restore normal haematopoiesis and/or to treat malignancies, by eradicating leukaemic cells recognised by donor T cells. This is referred to as graft-versus leukaemia (GvL) effect, which provides the chance of reducing the risk of disease relapse (Horowitz et al., 1990; Weiden et al., 1979). However, this effect is counterbalanced by a life-threatening complication, termed graft-versus-host disease (GvHD). GvHD is mediated by

immunocompetent donor T cells, which recognise the recipient (host) as foreign and induce an immune response to attack the host tissue and organs (Zeiser and Blazar, 2017). GvHD can be divided into acute and chronic GvHD and was previously distinguished according to the time point of disease manifestation: acute GvHD (aGvHD) was classified as symptom occurrence within 100 days after allo-HCT and chronic as symptom occurrence 100 days after allo-HCT. The manifestation of classic aGvHD symptoms after 100 days of transplantation can be subclassified into late-onset, recurrent or persistent aGvHD. However, this classification was refined by the National Institutes of Health (NIH). Therefore, GvHD is distinguished into acute and chronic based on clinical manifestations rather than the time of symptomatic onset after transplantation (Filipovich et al., 2005). While chronic GvHD can affect any organ in the body, aGvHD predominantly targets the skin, gastrointestinal tract and liver and bears symptoms like maculopapular rash, hyperbilirubinemia with jaundice, nausea, vomiting, anorexia and watery or bloody diarrhea with severe abdominal pain. Amongst all allo-HCT patients, 30-50% develop aGvHD, whose severity can differ from grade I (mild) to grade IV (very severe), based on the quantification of the symptoms (Glucksberg et al., 1974). Risk factors include degree of HLA disparity, donor and recipient sex disparity (female donor to male recipient), stem cell source, intensity of conditioning regimen and type of GvHD prophylaxis (Flowers et al., 2011; Gale et al., 1987; Jagasia et al., 2012; Kollman et al., 2001; Loiseau et al., 2007). The pathophysiological mechanism of aGvHD (Figure 1.3) can be divided into three phases: the initiation phase, T cell activation and the effector phase (Malard et al., 2023). The initiation of aGvHD is driven by the allo-HCT conditioning regimen (chemotherapy and/or radiotherapy), which leads to the damage of host tissues, especially the intestinal mucosa. This tissue damage results in the release of DAMPs (e.g. high-mobility group box 1 (HMGB1), uric acid, adenosine triphosphate (ATP), heat shock proteins, IL-33, heparan sulphate and proteoglycans) (Toubai et al., 2016) but also PAMPs, such as lipopolysaccharide (LPS), due to the translocation of microbiota (Hill et al., 1997). PAMPs and DAMPs lead to the activation of antigen-presenting cells (DCs, macrophages, B cells), which further induce the activation of donor T cells.

Activated alloreactive CD8⁺ T cells then target tissues via induction of apoptosis through FAS-FAS-ligand interaction and the release of granule contents, including granzyme B and perforin, while activated alloreactive CD4⁺ T cell-targeted cell damage is mediated by pro-inflammatory cytokines, such as IFN γ and tumour necrosis factor α (TNF α) (Baker et al., 1996; Graubert et al., 1997; Borsotti et al., 2007). The recognition of host antigens on host APCs by donor T cells is usually sufficient to initiate T cell-mediated aGvHD. However, donor-derived APCs can augment CD8⁺ T cell-mediated aGvHD via cross-presentation (Duffner et al., 2004; Matte et al., 2004; Shlomchik et al., 1999).

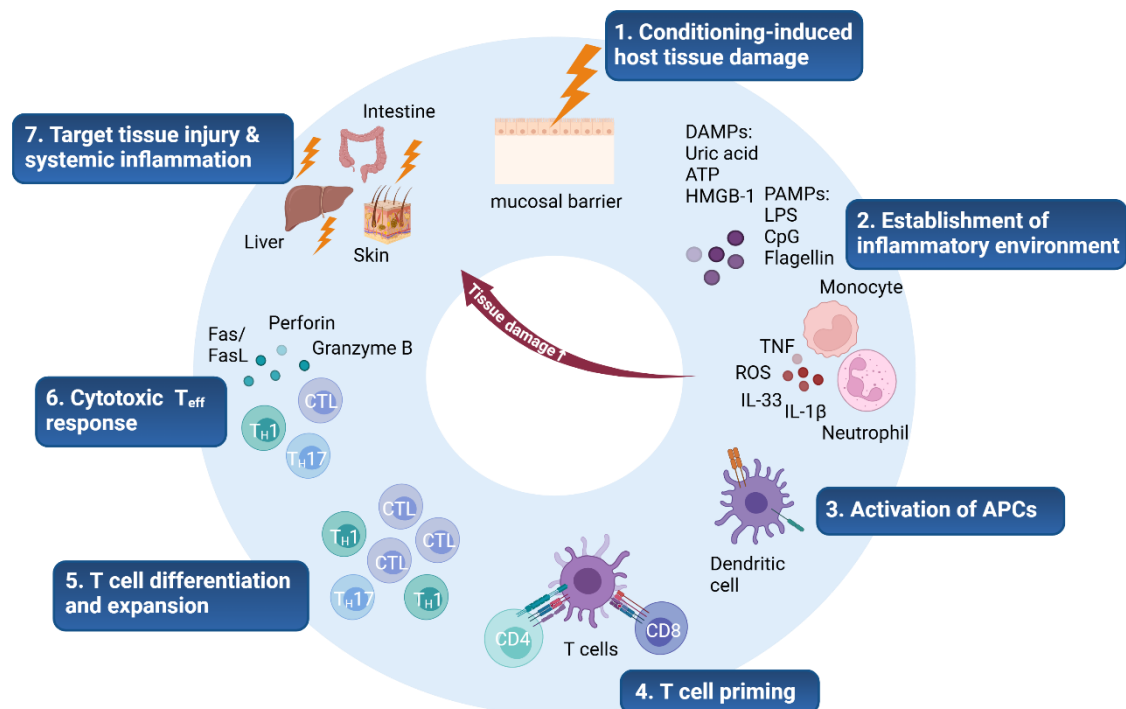


Figure 1.3 | Graft-versus-Host-Disease pathophysiology. The conditioning regimen induces tissue damage in mucosal barrier sites (1), which results in the release of damage-associated molecular patterns (DAMPs) from damaged cells and translocation of bacteria through the destructed epithelial barrier. Pathogen-associated molecular patterns (PAMPs) and DAMPs lead to the activation of local tissue-resident and innate immune cells, resulting in cytokine production and immune cell infiltration (2). Antigen-presenting cells (APCs), such as dendritic cells (DCs) become activated (3) and migrate into secondary lymphoid organs (SLOs) for donor T cell priming (4). Following differentiation and expansion (5), allo-reactive donor T cells migrate to target organs to induce cytotoxic effector responses (6), promoting further tissue damage (7). ATP: adenosine

triphosphate, CTL: cytotoxic T lymphocyte, HMGB-1: high mobility group box 1 protein, IFN: interferon, IL: interleukin, LPS: lipopolysaccharide, ROS: reactive oxygen species, T_{eff}: effector T cell, T_H: T helper cell, TNF: tumor necrosis factor. Figure modified according to Jacobsohn and Vogelsang, BioMed Central (2007) using Biorender.

1.2.2.1 The role of dendritic cells in acute GvHD

In the setting of allo-HCT, DCs play an important role in the initiation of donor T cell-mediated GvHD in the liver, intestine and skin (MacDonald et al., 2013). The conditioning regimen prior to allo-HCT leads to host tissue damage and the release of pro-inflammatory cytokines, DAMPs and PAMPs that cause DC activation through TLR stimulation. Activated host DCs present host antigens to CD4⁺ and CD8⁺ T cells, thus promoting their proliferation and differentiation into alloreactive effector cells (Zhang et al., 2002). In addition, donor cDCs have been reported to amplify alloreactive T cell responses via cross-presentation of host antigens (Markey et al., 2009). cDC1s are the main producers of IL-12. Alloreactive T cell responses are shaped by cDC1-derived IL-12 secretion, promoting IFN γ production in host-reactive T cells (Koyama et al., 2015). Moreover, host DCs expressing the Notch receptor ligand DLL4 revealed a greater ability to stimulate the generation of alloreactive T cells, indicating that alloreactive T cell responses are driven by the Notch signalling pathway (Meng et al., 2016; Mochizuki et al., 2013; Mochizuki et al., 2016).

DCs are crucial players in mediating central and peripheral tolerance (Audiger et al., 2017). Despite their important role in the initiation of GvHD onset, both host and donor DCs may contribute to the induction of donor T cell tolerance against host tissues after allo-HCT. Teshima et al. (2002) reported that FLT3L treatment of recipient mice causes the expansion of CD8 α ⁺ DCs that can suppress donor T cell responses *in vivo*, resulting in less severe GvHD (Teshima et al., 2002). DCs inhibit autoreactive T cells through the interaction of PD-L1 and CD80/86 with the inhibitory molecules PD-1 and CTLA-4 expressed on T cells (Blazar et al., 2003). Moreover, the transfer of host CCR9⁺ pDCs as well as donor pre-pDCs was shown to inhibit or attenuate GvHD in mice receiving MHC-mismatched donor T

cells, while retaining the GVL effect (Hadeiba et al., 2008; Hassan et al., 2019; Lu et al., 2012). pDCs and pre-pDCs can promote T_{reg} expansion and function and suppress antigen-specific immune responses, thus providing a tolerogenic effect on the induction of donor T cells against host tissues (Lu et al., 2012).

Preclinical studies revealed that allo-HCT is associated with a deficit in all DC subsets, correlating with the occurrence of severe GvHD (Banovic et al., 2009; Reddy et al., 2004; Wikstrom et al., 2015). Shono et al. (2010) have shown that GvHD impairs the reconstitution of DCs, as GvHD-associated inflammatory responses damage the BM niche, which leads to dramatically decreased haematopoiesis, including the reduction of CDPs (Shono et al., 2010). Impaired reconstitution of DCs is associated with an increased risk of infections (Zeiser and Blazar, 2017). Thus, the reconstitution of DCs following allo-HCT provides an effective strategy to re-establish protective immunity.

Given their important role in mediating donor T cell tolerance, the administration of tolerogenic DCs provides a promising strategy for enhancing GvL response and reducing the severity of GvHD.

1.2.2.2 Clinical strategies for acute GvHD treatment

Currently, several prophylactic treatment options for aGvHD have been developed. Pharmacological prophylaxis is based on the inhibition of calcineurin (an enzyme important for T cell activation) combined with the anti-metabolite drug methotrexate (Storb et al., 1986, Storb et al., 1989). Given the important role of T cells in aGvHD, T cell depletion (TCD) as a cell-based prophylactic strategy is another approach of significant interest. However, although *ex vivo* TCD is highly effective for GvHD prophylaxis, it is associated with a high rate of complications, including infections and disease relapse (Urbano-Ispizua et al., 1997; Wagner et al., 2005). Despite prophylaxis, aGvHD is treated first-line with glucocorticoids (Mielcarek et al., 2015). However, only about half of the patients respond to steroids (Saliba et al., 2007). Recently, the Janus kinase 2 (JAK2) inhibitor ruxolitinib was approved as the only standard second-line treatment for steroid-

refractory or steroid-dependent aGvHD based on a phase III clinical trial (Zeiser et al., 2020). Ruxolitinib reduces GvHD by inhibiting the production of pro-inflammatory cytokines, thus reducing T cell proliferation and preserving the benefit of the GvL effect (Choi et al., 2014; Spoerl et al., 2014). Patients with steroid-resistant and ruxolitinib-resistant aGvHD can only be treated by the use of one of the prophylactic treatment options (e.g. anti-thymocyte globulin, mycophenolate mofetil, alemtuzumab, anti-TNF, anti-IL2R, mammalian target of rapamycin (mTOR) kinase inhibitor, extracorporeal photopheresis, methotrexate, or faecal microbiota transplantation) (Malard et al., 2023).

At present, therapies targeting immune cells, such as T_{regs}, mesenchymal stromal cells (MSCs) and tolerogenic DCs (tDCs) to inhibit GvHD are under investigation. Induction of alloantigen-specific T_{regs} revealed a potential effect in preventing GvHD by inducing long-term tolerance without comprising general immunity and graft-vs-tumour activity (Zheng et al., 2013). Infusion of MSCs was well-tolerated and safe for patients. However, no evidence of efficacy in the prevention or treatment of aGvHD and no significant effect on relapse rate or overall survival was observed (Fisher et al., 2019; T. Li et al., 2021; L. Zhao et al., 2019; Morata-Tarifa et al., 2020). Placenta-derived decidual stromal cells (DSCs) represent a novel therapeutic target for aGvHD after allo-HCT, as these cells are more immunosuppressive than MSCs and DSC treatment resulted in a significant enhancement of the one-year survival rate of aGvHD (Baygan et al., 2017; Ringden et al., 2018). As previously mentioned, the activation of DCs plays an important role in the initiation of GvHD. However, tolerogenic DCs are also essential for the induction of immunological tolerance (Torres-Aguilar et al., 2010; Boks et al., 2012). The use of tDCs as GvHD treatment revealed great potential in preclinical studies, as administration of (autologous) tDCs induces antigen-specific graft tolerance and long-term allograft survival (Hill et al., 2011; Yang et al., 2013).

To date, most therapeutic strategies have directly been targeting T cells. However, targeting other immune cells, such as T_{regs}, MSCs and tDCs reveals a promising strategy to treat/prevent GvHD. Especially, the critical role of DCs in

mediating both immune activation and immune tolerance highlights the need for a better understanding of DCs as regulators in aGvHD to improve current or develop new therapeutic approaches.

1.3 The PD-1/PD-L1 axis and its functional role in immunity

T cell-based adaptive immunity has evolved for the recognition and destruction of aberrant cells such as pathogen-infected cells and cancer cells, but also self-antigens can be recognised and attacked by the immune system. This highlights the need for a tight balance between the induction of efficient immune responses and tolerance towards self-antigens to avoid self-damage caused by the immune system attacking host tissues (autoimmunity). The adaptive immune response is tightly controlled by co-stimulatory and co-inhibitory molecules, so-called immune checkpoints, that regulate the functional activity of antigen-specific T cells. To date, several immune checkpoint molecules have already been identified, including PD-1 (Ishida et al., 1992), CTLA-4 (Linsley et al., 1994), TIM-3 (Monney et al., 2002), LAG-3 (Baixeras et al., 1992) and many more. Besides their function in avoiding autoimmunity, immune checkpoints have been discovered as potential targets for anti-cancer strategies. Among those, the PD-1-PD-L1 axis is the most well-characterised immune checkpoint pathway that has been widely explored as clinical cancer immunotherapy in a large number of malignancies (Meng et al., 2024).

1.3.1 PD-1/PD-L1 signalling

The immune-inhibitory receptor PD-1 (CD279) represents a negative regulator of the immune system by inhibiting T cell immune function through the interaction with its two ligands PD-L1 (CD274) and PD-L2 (CD273) (Freeman et al., 2000; Latchman et al., 2001). PD-1 is expressed on different kinds of immune cells, including T cells, B cells, NK cells, monocytes and DCs (Keir et al., 2008). While PD-L1 is constitutively expressed on various immune cells such as T- and B cells, macrophages, DCs, MSCs and mast cells, as well as non-haematopoietic tissues

and tumour cells, PD-L2 is mainly restricted to macrophages, DCs and mast cells (Sharpe et al., 2007). Although PD-L2 binds to PD-1 with a three-fold higher affinity than PD-L1, PD-L1 is reported to be the primary ligand of PD-1 (Qin et al., 2019).

PD-1 and its ligands are type I transmembrane proteins belonging to the immunoglobulin (Ig) superfamily. PD-1 consists of an Immunoglobulin V (Ig-V)-like extracellular domain, a transmembrane region and a cytoplasmic domain harbouring two tyrosine-based signalling motifs, the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). The structure of PD-L1 is similar to PD-1 and contains an Ig-V- and Ig-C-like extracellular domain, a transmembrane domain and a short cytoplasmic tail (Keir et al., 2008).

PD-L1 binding to PD-1 leads to the phosphorylation of ITSM by Src family kinases and the recruitment of SHP1 and SHP2 protein tyrosine phosphatases. SHP1/2 recruitment causes the dephosphorylation of the TCR and CD28 signalling molecules, inhibiting T cell activation signalling. Inhibitory signalling results in the inactivation of the two major downstream signalling pathways, the phosphoinositide 3-kinase (PI3K)/AKT and Ras-MEK-ERK pathways (Sheppard et al., 2004; Yokosuka et al., 2012). PD-L1 ligation to PD-1 on T cells leads to different functional outcomes. In effector T cells, engagement of PD-1 by PD-L1 suppresses TCR or co-stimulatory signalling, resulting in inhibition of T cell proliferation, secretion of inflammatory cytokines and induction of apoptosis (Butte et al., 2007; Dong et al., 1999; Freeman et al., 2000; Keir et al., 2006; Latchman et al., 2004). In contrast, PD-L1 binding to PD-1 on T_{regs} induces T_{reg} proliferation (Francisco et al., 2009).

Besides its interaction with PD-1, it has been reported that PD-L1 can also interact with CD80 as a second receptor, triggering inhibitory signalling (Butte et al., 2007; Park et al., 2010). These findings were confirmed by Sugiura et al. (2019), showing that PD-L1 on APCs can interact with CD80 *in cis*, thus preventing the interaction between PD-L1 and PD-1.

1.3.2 Clinical relevance of the PD-1/PD-L1 axis

Under physiological conditions, the interaction between PD-1 and PD-L1 has been reported to play a critical role in the regulation of peripheral tolerance and autoimmunity. The PD-1/PD-L1 pathway provides tolerance to host tissues by promoting the development and function of T_{regs} and inhibiting the generation and activation of self-reactive T cells (Francisco et al., 2009; Keir et al., 2006; Probst et al., 2005). The interaction between PD-1 and PD-L1 is required to limit immune responses to avoid excessive immune-mediated tissue damage. The role of the PD-1/PD-L1 axis in autoimmunity was demonstrated in PD-1 knockout (KO) mice, where the breakdown of peripheral tolerance resulted in negative regulation of lymphocyte activation. PD-1^{-/-} mice were shown to more frequently develop lupus-like disease (C57BL/6), type I diabetes (non-obese diabetic (NOD) background), and dilated cardiomyopathies (BALB/c) (Ansari et al., 2003; Nishimura et al., 2001, 1999). Furthermore, in some experimental animal models for diabetes, experimental autoimmune encephalomyelitis (EAE) and autoimmune enteritis, blockade of PD-1/PD-L1 increased disease onset and severity (Pauken et al., 2013; Salama et al., 2003). Therefore, activation of the PD-1/PD-L1 immune checkpoint represents a potential target for the treatment of autoimmune diseases. Administration of PD-L1 Ig recombinant fusion protein, PD-1-specific toxins or Fc-PD-L1 has been shown to reduce disease severity of several inflammatory diseases, including rheumatoid arthritis, colitis, EAE, SLE and psoriasis (Qin et al., 2019). Moreover, the PD-1/PD-L1 pathway is critical for the progression of GvHD, where tolerance between the donor transplant and host tissues is pivotal (Blazar et al., 2003).

Several microorganisms causing chronic infection can exploit the PD-1/PD-L1 pathway to evade the immune system. Viruses causing chronic infections render virus-specific T cells non-functional due to persistently high levels of antigen exposure, causing T cell exhaustion. A main characteristic of exhausted CD8⁺ T cells is the high expression of PD-1, which is not required for the induction of T cell exhaustion but rather for its maintenance (Odorizzi et al., 2015). The functional role of PD-1/PD-L1 in chronic infection was first demonstrated in

lymphocytic choriomeningitis virus infection (Barber et al., 2006). Here, blockade of the PD-1/PD-L1 pathway using monoclonal antibodies restored virus-specific CD8⁺ T cell proliferation, cytokine production and cytotoxic capacity, resulting in a decreased viral load (Barber et al., 2006). Similar results were obtained in studies of simian immunodeficiency virus, hepatitis B and hepatitis C virus (Chen et al., 2007; Fuller et al., 2013; Velu et al., 2009). Besides viral chronic infection, the PD-1/PD-L1 pathway has additionally been demonstrated to play a key role in the chronicity of several bacterial and parasitic infections (e.g. *Helicobacter pylori*, *Schistosoma mansoni*, *Plasmodium falciparum*) (Beswick et al., 2007; Butler et al., 2012; Smith et al., 2004).

Given its role in T cell suppression, the PD-1/PD-L1 axis has gained great attention as a promising target for antibody-based anti-cancer approaches. PD-L1 is expressed on various cancer types (Fay et al., 2015; Jacobs et al., 2009; Katsuya et al., 2015; Nakanishi et al., 2007; Nomi et al., 2007; Strome et al., 2003; Wilmotte et al., 2005). Additionally, PD-L1 expression was demonstrated on tumour-infiltrating cells such as DCs, MDSCs and TAMs. Similar to chronic infection, the PD-1/PD-L1 pathway is used by cancer cells to escape the attack from the immune system (Blank et al., 2004; Taube et al., 2012). It has been reported that PD-L1 protects cancer cells from direct attack by cytotoxic T lymphocytes (CTLs). The binding of PD-L1 expressed on cancer cells or tumour-infiltrating cells to its receptor PD-1 expressed on activated CD8⁺ T cells leads to the inhibition of TCR and co-stimulatory signalling cascades, resulting in T cell anergy and apoptosis (Butte et al., 2007; Dong et al., 2002; Freeman et al., 2000). To overcome T cell suppression and boost the anti-tumour immunity, monoclonal antibodies targeting immune checkpoints have been extensively studied. Several antibody-based inhibitors targeting the PD-1/PD-L1 axis (nivolumab, pembrolizumab, avelumab, atezolizumab, durvalumab) have been approved by the Food and Drug Administration (FDA) for the treatment of melanoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma, bladder cancer, colorectal cancer (CRC), Hodgkin lymphoma and various other cancer types (Wu et al., 2021). Although these therapies revealed clinical success, a proportion of

patients failed to respond, which can be explained by the diversity of the TME. Current treatment approaches focus on combination therapies that encounter tumour heterogeneity. A combination of the checkpoint inhibitors anti-PD1 (nivolumab) and anti-CTLA-4 (ipilimumab) has already been approved by the FDA due to improved clinical response rates (Cella et al., 2019; Larkin et al., 2015). Further strategies for combination therapies with PD-1/PD-L1 blockade are under investigation, including blockade of other inhibitory receptors and immunoregulatory cytokines, administration of agonists of co-stimulatory molecules and treatment with homeostatic cytokines or engineered T cells (Sharma and Allison, 2015).

The diverging functions of the PD-1/PD-L1 axis in various clinical implications, including tolerance, autoimmunity, chronic infections and cancer, highlight the need for a better understanding of the diverse immunoregulatory roles of this pathway in individual disease settings to optimise clinical outcomes.

1.3.3 Molecular regulation of PD-L1 expression

PD-L1 is expressed on various immune cells, non-haematopoietic cells and tumour cells. Expression of PD-L1 on these various cell types can be modulated by several different mechanisms, including transcriptional regulation, post-transcriptional regulation and post-translational modification (Sun et al., 2018a) (Figure 1.4).

PD-L1 gene expression is controlled by inflammatory signalling. IFN- γ can be generated by activated T cells and NK cells and is among the inflammatory cytokines the most prominent inducer of PD-L1 expression (Abiko et al., 2015). IFN- γ signalling results in the activation of the JAK-STAT pathway, inducing the expression of transcription factors, so-called interferon-responsive factors. Among those factors, IRF1 is the most important TF in the IFN- γ signalling pathway, and has been shown to activate PD-L1 expression through binding to IRF1 response elements in the PD-L1 gene promoter (Lee et al., 2006; Yan et al., 2020). Besides IFN- γ , type I IFNs, such as IFN- α and IFN- β , can also induce PD-

L1 expression in different cell types, including melanoma, endothelial cells, monocytes and DCs (Eppihimer et al., 2002; Garcia-Diaz et al., 2019; Schreiner et al., 2004). Other inflammatory stimuli linked to induced PD-L1 expression include TLR agonists, such as LPS (TLR4) and polyinosinic:polycytidylic acid (poly I:C, targeting TLR3) (Huang et al., 2013; Mezzadra et al., 2017; Pulko et al., 2009) and various pro- and anti-inflammatory cytokines, like IL-1 β , IL-4, IL-6, IL-10, IL-17, IL27, TNF- α , transforming growth factor (TGF)- β (Sun et al., 2018a).

In addition to inflammatory signalling, PD-L1 expression can also be regulated by oncogenic signalling. Several oncogenic transcription factors have been identified that induce PD-L1 transcription through direct binding to the PD-L1 promoter. These TFs include the oncogene Myc, signal transducer and activator of transcription 3 (STAT3) and NF κ B (Atsaves et al., 2017; Casey et al., 2016; Gowrishankar et al., 2015; Huang et al., 2013; Marzec et al., 2008; J. Wang et al., 2017). Furthermore, under hypoxic conditions, hypoxia-inducible factor (HIF)-1 α and HIF-2 α directly bind to the hypoxia response element in the PD-L1 promoter region, thus inducing PD-L1 expression in tumour (Barsoum et al., 2014; Messai et al., 2016; Noman et al., 2014). In addition to oncogenic TFs, also signalling pathways that are frequently activated in cancer, such as the PI3K/AKT and MEK-ERK signalling pathways, have been shown to increase PD-L1 transcription (Atefi et al., 2014; Liu et al., 2007; Song et al., 2013). Moreover, genetic alterations and epigenetic modifications (e.g. histone acetylation, histone methylation) can drive PD-L1 expression in cancer (Chen et al., 2017, 2015; Darvin et al., 2019; Ota et al., 2015; Wang et al., 2020).

On the post-transcriptional level, PD-L1 can be regulated by various microRNAs (miRNAs) that suppress PD-L1 translation through direct binding to the 3' untranslated region (UTR) of the PD-L1 mRNA (Cortez et al., 2016; Wang et al., 2015; Yee et al., 2017). Besides the direct inhibition, there is extensive evidence that miRNAs can also positively modulate PD-L1 in an indirect manner by promoting STAT3 phosphorylation or inhibiting phosphatase and tensin homolog (PTEN) expression, which in turn leads to increased PD-L1 expression (D. Tang et al., 2018; Zhu et al., 2014).

PD-L1 protein level can be modulated by various post-translational modifications (PTMs), such as phosphorylation, glycosylation and ubiquitination (Li et al., 2020). In recent studies, several positive and negative regulators for post-translational regulation of PD-L1 have been identified. Ubiquitination is a crucial PTM that reversibly labels target proteins for degradation. One positive regulator of PD-L1 expression is CKLF-like MARVEL transmembrane domain-containing protein 6 (CMTM6). CMTM6 can bind to PD-L1 and prevent its ubiquitination and degradation. The regulation of PD-L1 levels by CMTM6 has been observed in various cancer types, including melanoma, NSCLC, breast cancer, pancreatic cancer and CRC (Burr et al., 2017; Mezzadra et al., 2017). A study by Zhang et al. (2017a) in human cervical cancer and breast cancer cells demonstrated that the cyclin-dependent kinase 4 (CDK4) complex negatively affects PD-L1 protein expression by indirectly regulating its ubiquitination and degradation through phosphorylation and activation of the cullin 3-SPOP E3 ubiquitin ligase (Zhang et al., 2018). Deubiquitinases can prevent protein degradation by removing attached ubiquitin chains, hence stabilising targeted proteins. Several deubiquitinases have been identified to play a role in PD-L1 regulation, such as the COP9 signalosome 5 (CSN5), which was described to remove ubiquitin chains from PD-L1 in breast cancer cells, leading to increased PD-L1 protein levels (Lim et al., 2016). Moreover, ubiquitin-specific peptidase 9, X linked (USP9x) and USP22 have been demonstrated to deubiquitinate and stabilise PD-L1 protein (Huang et al., 2019; Jingjing et al., 2018).

Another important PTM of PD-L1 is glycosylation. N-glycosylation of PD-L1 has been reported to be necessary for the binding of PD-L1 to PD-1 (Li et al., 2018). N-glycosylation by N-linked glycosyltransferases such as SST3 stabilises PD-L1, thus preventing its degradation (Hsu et al., 2018). This was confirmed by another study showing that phosphorylation of non-glycosylated PD-L1 by glycogen synthase kinase 3 β (GSK-3 β) promotes PD-L1 instability and induces PD-L1 proteasomal degradation (Li et al., 2016).

Current mechanisms of action of PD-1/PD-L1 targeting agents focus on the effect on the activity of PD-1 expressing T cells. However, the potential role of PD-L1

signalling in promoting tumour cell survival and the multiple processes contributing to PD-L1 regulation highlight the need to additionally focus on the processes that regulate PD-L1 expression and PD-L1 signalling to improve clinical responses to current therapeutic approaches in anti-cancer immunity.

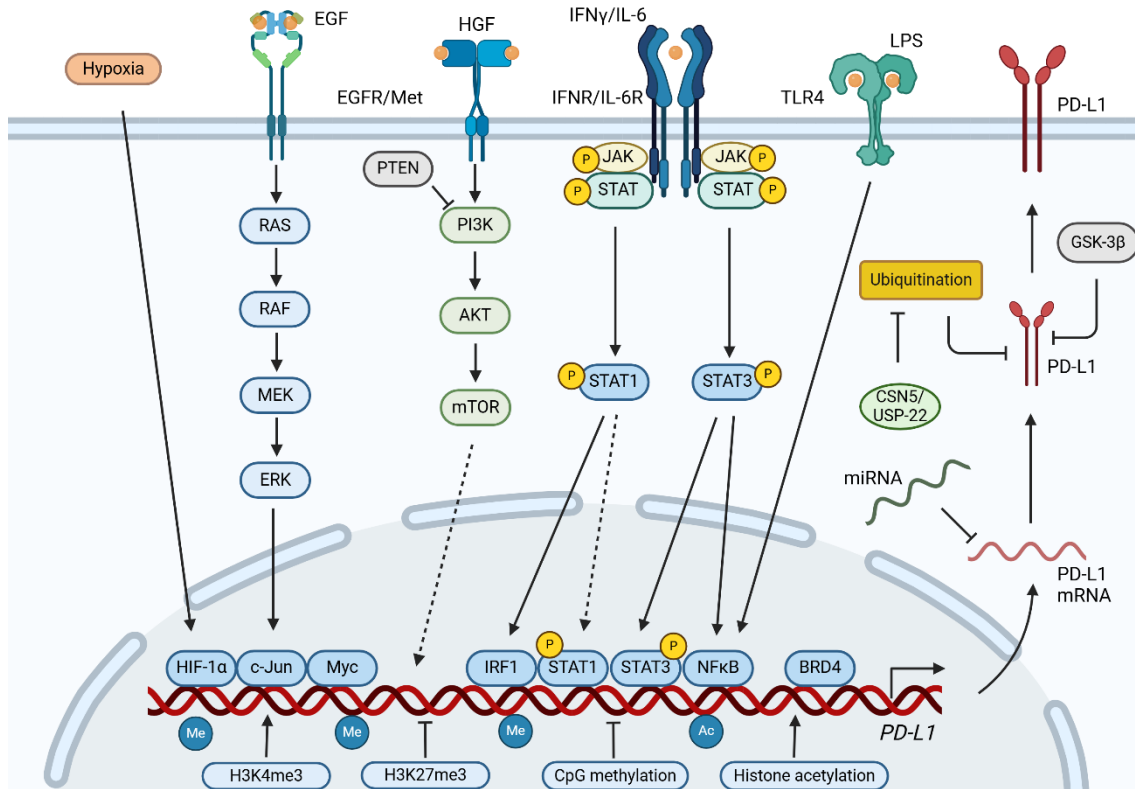


Figure 1.4 | Mechanisms regulating PD-L1 expression. PD-L1 expression is regulated on the transcriptional level (inflammatory stimuli, oncogenic signalling, transcription factors), post-transcriptional level (miRNAs) and post-translational level (ubiquitination, phosphorylation, glycosylation) as well as by genomic alterations and epigenetic modifications (histone methylation, histone acetylation). EGFR: epidermal growth factor receptor, HGF: hepatocyte growth factor, IFN: interferon, IL: interleukin, TLR: toll-like receptor, LPS: lipopolysaccharide, PI3K: phosphoinositide-3-kinase, GSK-3 β : glycogen synthase kinase 3 β , CSN5: COP9 signalosome 5, IRF: interferon, STAT: signal transducer and activator of transcription. NF κ B: nuclear factor κ -B, HIF-1 α : hypoxia-inducible factor-1 α , PTEN: phosphatase and tensin homolog, mTOR: mammalian target of rapamycin, H3K4me3: tri-methylation of histone H3 on lysine 4. Figure created with Biorender modified to Yi et al., Journal of Hematology & Oncology, 2021.

1.3.4 The function of PD-L1 on dendritic cells

Antigen presentation plays a critical role in an effective anti-tumour immune response. Since cDC1s can cross-present tumour antigens to CD8⁺ T cells, DC are the most important APCs in the TME, inducing anti-tumour immune responses by mediating T cell priming and activation (Roberts et al., 2016; Hildner et al., 2008). However, it has been reported that in the TME, infiltrating DCs can switch from an immunostimulatory to an immunosuppressive state (Norian et al., 2009; Scarlett et al., 2012; Strauss et al., 2020). In the TME, tumour cells and immune cells such as DCs, macrophages, MDSCs and T_{regs} highly express the inhibitory immune checkpoint molecule PD-L1 (Nguyen and Ohashi, 2015). However, the contribution of PD-L1 on these cells is not completely understood yet. In recent years, PD-1/PD-L1 blockade therapy has emerged as a promising treatment strategy against many types of cancer (Brahmer et al., 2012; Herbst et al., 2014; Topalian et al., 2012). Interestingly, the clinical response to anti-PD-L1 blockade was strongest when PD-L1 was expressed by tumour-infiltrating immune cells rather than by tumour cells (Herbst et al., 2014). This is consistent with several studies describing that PD-L1 expressed on immune cells plays an important role in inhibiting anti-tumour immune responses (Antonios et al., 2017; Kleinovink et al., 2017; Lin et al., 2018; Rashidian et al., 2019; H. Tang et al., 2018). In a recent study, Peng et al. (2020) described that tumour-infiltrating DCs express high levels of PD-L1, which plays a critical role in limiting anti-tumour immune responses *in vivo*. They observed that the therapeutic effect of PD-L1 blockade therapy was completely gone in DC-conditional knockout mice, even though other cells still expressed high levels of PD-L1. This leads to the suggestion that PD-L1 on DCs plays a key role in response to PD-L1 blockade therapy (Peng et al., 2020). Similar observations were obtained by Oh et al. (2020), who found that tumour growth in mice lacking PD-L1 on DCs could be effectively inhibited and the cytotoxic capability of CD8⁺ T cells was enhanced. In addition, Oh et al. (2020) demonstrated the key role of cDC1s in PD-L1 blockade therapy by showing that tumour growth was not significantly inhibited in cDC1-deficient mice (BATF3^{-/-}) receiving anti-PD-L1 treatment.

Under physiological conditions, PD-L1 expression protects the host from autoimmune diseases by inhibiting non-specific activation or killing by CTLs (Keir et al., 2006; Probst et al., 2005). Although DCs are the main APC presenting tumour antigens to T cells, it has been shown that upregulation of PD-L1 protects DCs from CTL killing, but in turn dampens the anti-tumour immune response (Chen et al., 2018; Peng et al., 2020). PD-L1 upregulation on DCs was found to be mediated by IFN- γ produced by CD8⁺ T cells and activated T cells, confirming IFN- γ as one of the most potential inducers of PD-L1 expression (Peng et al., 2020). Additionally, in ovarian cancer TME, PD-L1 expression on myeloid DCs was induced by IL-10 and VEGF (Curiel et al., 2003). Blocking PD-L1 on DCs significantly promotes the proliferation and granzyme B production by CD8⁺ T cells, enhancing T cell effector ability (Mayoux et al., 2020).

Besides PD-1, PD-L1 was additionally found to engage with CD80. Interestingly, DCs are capable of expressing PD-1, PD-L1 and CD80 at the same time (Zhao et al., 2018). PD-L1 on DCs can interact with CD80 in *cis*, concurrently preventing PD-L1/PD-1 and CD80/CTLA-4 binding, without affecting CD80 association with CD28 (Sugiura et al., 2019; Y. Zhao et al., 2019; Zhao et al., 2018). However, further investigations revealed that PD-L1 is expressed much more abundantly on tumour-associated DCs compared with CD80 (Mayoux et al., 2020). Under these conditions, PD-L1 may sequester CD80 on DCs in *cis*, but free PD-L1 is still available for PD-1 binding to inhibit T cell activation. Anti-PD-L1 antibodies are capable of blocking both PD-L1/PD-1 and PD-L1/CD80 *cis* interactions. Those antibodies enhance the interaction between CD80 and CD28, which promotes T cell immune responses (Mayoux et al., 2020). However, at the same time, they facilitate CD80 binding to CTLA-4, which leads to a dampened T cell activity (Zhao et al., 2019). Nevertheless, this side effect can effectively be avoided using a combination therapy of anti-PD-L1 and anti-CTLA-4 blockade.

The impact of PD-L1 on DCs is not limited to the regulation of T cell activation. Blockade of PD-L1 expressed on DCs was additionally reported to enhance DC maturation and their antigen-presentation capabilities (Curiel et al., 2003; Mayoux et al., 2020; Sun et al., 2019).

Based on the important role of PD-L1 on DCs in regulating anti-tumour immune responses, targeting PD-L1 regulation in DCs could provide a potential therapeutic strategy in anti-cancer immunity.

1.4 G-quadruplexes

Besides the canonical double helix (B-DNA), genomic DNA can fold into a variety of alternative conformations. Among these non-B structures are hairpins, cruciforms, triplexes, i-motifs and G-quadruplexes (G4s) (Bacolla and Wells, 2004). G4 structures can fold in sequence-specific genomic regions, which are evolutionarily conserved (Capra et al., 2010). Therefore, these structures and their biological relevance have been extensively studied in the past.

1.4.1 Structure and formation of DNA G-quadruplexes

G-quadruplex structures can form in guanine-rich regions of DNA and RNA. G4s arise from the stacking of G-quartets, which are formed by Hoogsteen hydrogen bonding of four guanines arranged within a planar array (Gellert et al., 1962). G4 structures can form in regions harbouring a specific consensus motif (G4-motif) composed of at least four runs of guanines, so-called G-tracts. The sequence motif was predicted to contain G-tracts with three or more guanines interrupted by loop sequences, which reveal up to seven nucleotides in length ($G_{\geq 3}N_{1-7}G_{\geq 3}N_{1-7}G_{\geq 3}N_{1-7}G_{\geq 3}$) (Huppert and Balasubramanian, 2005; Todd et al., 2005). However, recent studies have shown that G4 structures can also form from sequences that deviate from the consensus motif in the number of G-tracts and loop length ($G_{>2}N_xG_{>2}N_xG_{>2}N_xG_{>2}N_x$) (Mukundan and Phan, 2013; Sengar et al., 2019). G4s are stabilised by monovalent cations located within the centre of the G-quartets with a stabilising preference in the order $K^+ > Na^+ > Li^+$ (Figure 4A) (Sen and Gilbert, 1990). Furthermore, numerous factors including the number of G-tetrads, loop length and topology affect the stability of G4 structures (Bugaut and Balasubramanian, 2008; Hazel et al., 2004). Based on the strand direction and

loop orientation, three main types of G4 topologies can be distinguished, including a parallel, anti-parallel and hybrid form (Figure 4B). Additionally, further diversity in G4 structures is given through intra- or intermolecular folding of G-rich strands (Burge et al., 2006). However, the biological consequences of the various G4 topologies are not known, so far.

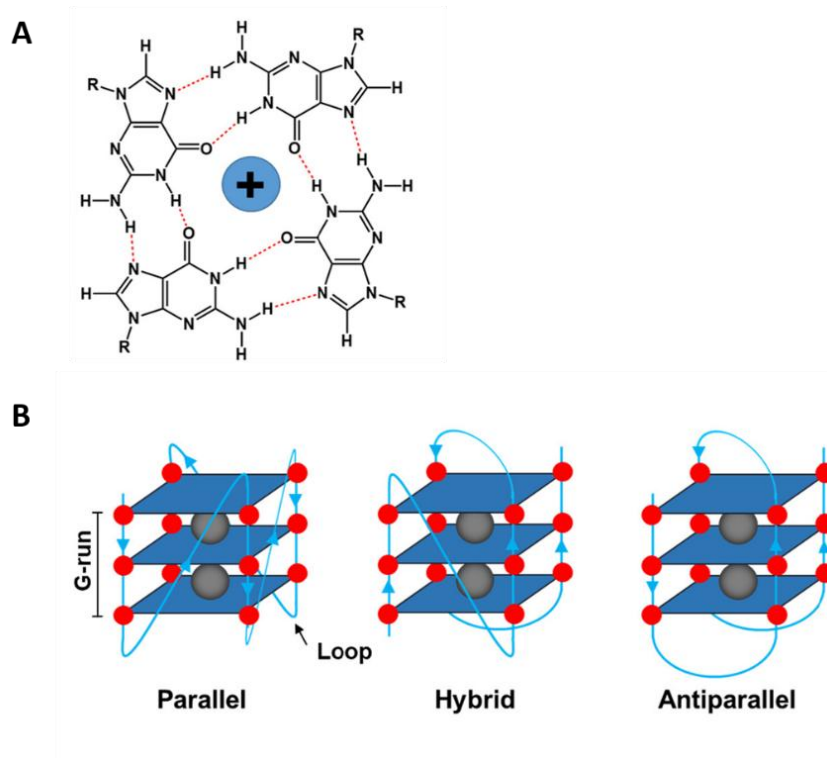


Figure 1.5 | Structural variety of G4 DNA. G-quadruplexes (G4s) form *in vitro* and *in vivo* in DNA or RNA sequences containing four G runs of at least two guanines. A) Four Guanines arranged within a planar quartet (G-quartet) are stabilised by Hoogsten hydrogen bonding. The planar G-quartets stack on top of one another, forming the G-quadruplex structure stabilised by monovalent cations (such as Na^+ , K^+) in their center. B) G-quadruplex structures can arise from intra- or intermolecular folding of the G-rich strands and can adopt parallel, anti-parallel and hybrid G4 topologies according to the orientation of the strands. Figure modified according to Frasson et al., International Journal of Biological Macromolecules, 2022.

1.4.2 Regulation and functions of DNA G-quadruplexes

Mapping of G4 structures using a combined method of G4-dependent DNA polymerase stalling and next-generation sequencing (G4-Seq) identified about

700,000 G4 motifs in the human and 790,000 G4 motifs in the mouse genome that are predicted to fold into G4 structures (Chambers et al., 2015; Marsico et al., 2019). However, not all sequences with G4 potential form structures in cells. It is suggested that G4 formation is distinct in different cell types or cell cycle stages. Computational studies in various organisms revealed a general enrichment of G4 motifs in regions associated with genome regulation, including telomeres, replication origins, transcriptional starting sites and double-strand break (DSB) sites (Bochman et al., 2012). The conserved localisation of G4 motifs across various species suggests potential functions of DNA G4 structures in several biological processes such as DNA replication, transcription and telomere maintenance. The relevance of G4 formation for those biological processes was elucidated through the identification of specific proteins that directly interact with G4 structures and regulate G4 formation, such as G4-unwinding helicases and several other natural G4-binding molecules (Mishra et al., 2016; Sauer and Paeschke, 2017; Simon et al., 2023). Furthermore, within the last years, several synthetic G4 ligands have been developed, which specifically bind to DNA G4s and modulate or stabilise their formation, thus facilitating the investigation of G4 formation and their impact on cellular functions *in vitro* and *in vivo*. To date, about 1000 small molecules targeting G4 structures have been reported (Li et al., 2013). Some well-known DNA G4 ligands include Braco-19, RHPS4, pyridostatin (PDS), PhenDC3, telomestatin, TMPyP4 (5,10,15,20-Tetrakis-(N-methyl-4-pyridyl) porphine) and CX-5461.

Telomeres are nucleoprotein complexes at the end of chromosomes essential to maintain genome integrity by protecting chromosomes from degradation and end-to-end fusion (Zakian, 2012). Telomeric DNA consists of 5'-TTAGGG-3' repeats showing a single-strand overhang at the end, which can form stable G4 structures *in vitro* (Moyzis et al., 1988; Williamson et al., 1989). Telomeric G4s were reported to impact telomere stability and the activity of telomerase, an RNA-reverse transcriptase complex that extends 3' ends of chromosomes to prevent telomere shortening and genome instability. G4 structures in telomeric DNA can act as a protective cap at chromosome ends through the binding of several

telomeric proteins such as Rap1-interacting factor 1 (RIF1) and telomeric repeat-binding factor 2 (TRF2) (Biffi et al., 2012; Kanoh et al., 2015; Smith et al., 2011). Furthermore, the formation of intramolecular anti-parallel telomere DNA G4s was shown to prevent telomere extension by limiting the access of telomeres to the telomerase. The complex POT1-tripeptidyl-peptidase 1 (POT1-TPP1), which is responsible for the recruitment and processivity of human telomerase, is known to destabilise G4s (Xu et al., 2021). However, in a recent study performed *in vitro*, Jansson et al. (2019) revealed that G4 folding within the active pocket of human telomerase supports POT1-TPP1-dependent telomerase processivity (Jansson et al., 2019), suggesting that G4 formation is important during human telomere DNA synthesis.

Computational studies predicted that G4 motifs are enriched in human gene promoters compared to the rest of the genome (Huppert and Balasubramanian, 2007), suggesting a potential role of G4s in transcriptional regulation. In a previous study, Hänsel-Hertsch et al. (2016) demonstrated G4 structures in chromatin mapped to regulatory regions upstream of the transcription starting site (TSS) of transcribed genes, further supporting the link between G4s and transcription. It has been reported that G4 stabilisation by G4-specific ligands can regulate transcription positively and negatively. The role of G4 structures as transcriptional regulators will be explained in more detail in section 1.4.3.

G4 structures have been postulated to play a biological role during replication. Mapping the genome-wide location of replication origins revealed an enrichment of predicted G4 sequences (PQS) in replication origins (Besnard et al., 2012), suggesting a supportive role for G4s in the initiation of DNA replication. During DNA replication, the two DNA strands of the DNA double helix are separated, resulting in a single-stranded conformation, which favours G4 structure formation. G4 structures that form during replication can act as an obstacle to DNA polymerase, resulting in replication fork stalling causing DNA double-strand breaks (Lopes et al., 2011; Paeschke et al., 2013).

Besides their biological functions in telomere maintenance, replication and transcriptional regulation, G4 structures are strongly associated with genomic instability. Uncontrolled formation or disruption of DNA G4 structures can lead to changes in cellular processes such as altered transcription, replication fork stalling or induced mutations, resulting in genome instability (Linke et al., 2021). To preserve genome stability, G4 structures are tightly regulated by various G4-interacting proteins, such as helicases (e.g. Pif1, FANJC, BLM, WRN) that specifically unwind DNA G4 structures within cells (Sauer and Paeschke, 2017; Schult et al., 2023). However, several studies demonstrated that in the absence of these helicases, G4 structures persist and are the driving cause of genome instability (Castillo Bosch et al., 2014; Dahan et al., 2018; De Magis et al., 2019; London et al., 2008; Paeschke et al., 2011). Furthermore, the stabilisation of G4 structures using G4-specific small molecules, such as PDS, causes replication-dependent and transcription-dependent DNA DSBs leading to genome instability (Rodriguez et al., 2012). Especially, cells compromised in their ability to process G4s are sensitive to G4-stabilising ligands (Wang et al., 2019a; Wu et al., 2018, 2008), highlighting the potential of G4 ligands as a therapeutic tool.

1.4.3 G-quadruplexes as transcriptional regulators

G4 motifs are enriched in nucleosome-depleted regions within 1 kb upstream of the TSS of protein-encoding genes (Hänsel-Hertsch et al., 2016; Huppert and Balasubramanian, 2007). About 50 % of human genes contain G4 motifs close to their promotor regions, suggesting a potential function of G4 structures in regulating gene expression (Huppert and Balasubramanian, 2007). Bioinformatic analysis revealed that promotor G4 motifs are enriched in human oncogenes and regulatory genes such as transcription factors, while they are under-represented in house-keeping and tumour suppressor genes (Eddy and Maizels, 2006; Huppert and Balasubramanian, 2007).

Increasing evidence highlights the implication of promoter G4s in regulating gene transcription. Initially, it was demonstrated *in vitro* that G4s can act as a physical

obstacle to RNA polymerase II processivity, causing transcriptional inhibition (Broxson et al., 2011). Several studies described that stabilisation of G4 structures in oncogenic promoters such as MYC, KRAS and KIT leads to reduced oncogene expression (Cogoi and Xodo, 2006; Siddiqui-Jain et al., 2002; Bejugam et al., 2007; Morgan et al., 2016), providing a potential target for anti-tumour therapeutic approaches. However, recent studies reported that G4 structures in promoters can also stimulate gene transcription (Chen et al., 2024; Esain-Garcia et al., 2024; Lago et al., 2021; Shen et al., 2021). The effect of G4s on regulating gene transcription depends on multiple factors, including the strand harbouring the G4 motif, recruitment of TFs, chromatin remodelling and genetic variations within the G4 forming sequence (Agarwal et al., 2014; Lago et al., 2021; Lorenzatti et al., 2023; Mao et al., 2018). Lorenzatti et al. (2023) reported that single nucleotide variants (SNVs) in G4s can contribute to the onset or susceptibility of human diseases by affecting *in vitro* G4 formation and stability of promoter G4s, which further modulates transcription of human disease-related genes. Furthermore, various studies describe that promoter G4s are primarily found within nucleosome-depleted, open chromatin and that this incidence is associated with active gene transcription (Esnault et al., 2023; Hänsel-Hertsch et al., 2016; Lago et al., 2021; Shen et al., 2021; Zyner et al., 2022). Based on these data, it is suggested that promoter G4s stimulate the recruitment of TFs and chromatin remodelling factors to favour open chromatin formation and initiate the transcription of highly active genes.

Computational studies focusing on TF binding sites revealed that they are proximal to or overlapping with G4 motifs in human gene promoters (Eddy and Maizels, 2008; Hou et al., 2019; Spiegel et al., 2021; Todd and Neidle, 2008). Spiegel et al. (2021) demonstrated that G4s in promoters can act as TF binding hubs, resulting in elevated gene expression through increased binding of TFs to promoter G4s followed by recruitment of RNA polymerase II. This hypothesis was further supported by the observation that several TFs (e.g SP1, LARK, cellular nucleic acid-binding protein (CNBP), activator protein 1 (AP-1)) display high affinity binding to G4s *in vitro* (Raiber et al., 2012; Niu et al., 2019; De Rache et

al., 2024; Borgognone et al., 2010; Lago et al., 2021) and that promoters containing G4 structures show higher RNA polymerase II occupancy than those without (Shen et al., 2021). Furthermore, TF recruitment to promoter G4s can enhance gene transcription by unfolding G4 structures as described for CNBP (David et al., 2019). In addition to TFs, transcription of genes containing promoter G4s could also be regulated through the binding of other G4-interacting proteins, such as nucleolin and NHM23H2. One study reported that *in vivo* binding of recombinant nucleolin to the G4 motif within the MYC promoter leads to G4 stabilisation and inhibition of MYC transcription by competing binding with TFs (González et al. 2009). In contrast, the binding of NHM23H2 to the G4 structure resulted in enhanced MYC gene transcription through the unfolding of the G4 structure (Thakur et al. 2009). Besides the direct binding of TFs, G4s can also have a cooperative role in transcription initiation by facilitating TF interaction with DNA due to a G4-mediated altered DNA methylation state (Jara-Espejo and Line, 2020; Mao et al., 2018).

Several studies also focused on the consequences of G4 formation on transcription elongation. Depending on which DNA strand encodes the G4 motif, the structure could either inhibit or enhance transcription. G4 motifs present on the template strand were observed to have an inhibitory effect on transcription, as the transcription machinery is blocked by the G4 structure. On the other hand, G4 structures forming on the non-template strand enhanced transcription levels by maintaining the transcribed strand in a single-stranded conformation, thus facilitating transcription re-initiation (Agarwal et al., 2014).

Taken together, G4 formation can positively and negatively impact gene expression. Most models linking G4s and transcription have been largely based on correlations and plasmid-based studies. Hence, additional work is needed to fully understand the molecular mechanisms underlying G4-modulated transcription in a chromatin context. Nevertheless, G4 formation itself is a dynamic process dependent on several factors, including protein interactions, cell type and stimuli, of which not all are known yet.

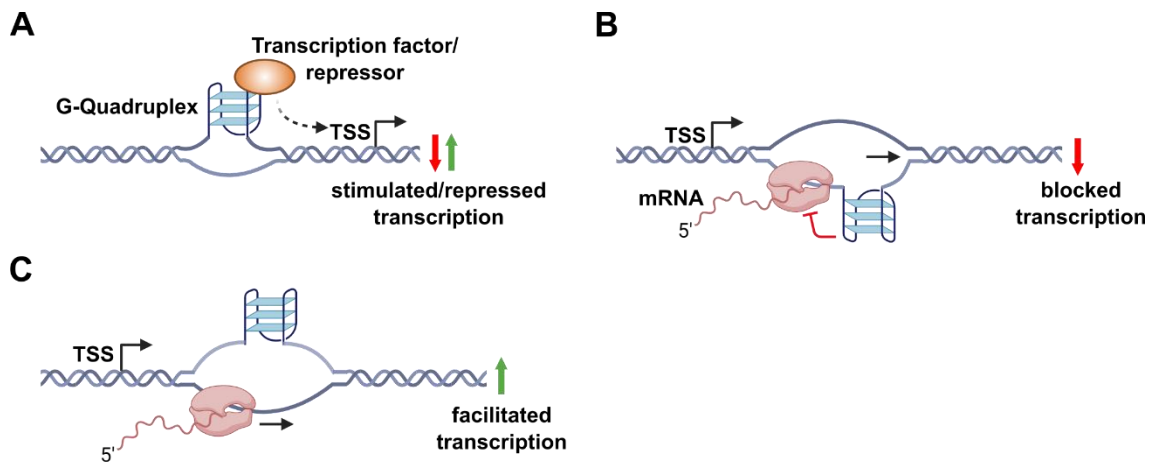


Figure 1.6 | The biological function of G-Quadruplexes on transcriptional regulation. A) G-quadruplex (G4) formation upstream of the transcription starting site (TSS) can result in the recruitment and binding of transcription factors or repressor proteins, which may either facilitate or restrain transcription. B) G4 formation on the template strand directly impedes RNA polymerase II processivity. C) G4 sequences on the non-template strand facilitate transcription by keeping the strands in single-stranded conformation. Figure created with Biorender.

1.4.4 G-quadruplexes as cancer immunotherapeutic target

Several studies demonstrated a higher presence of G4 structures in cancer compared to normal state (Biffi et al., 2014; Hänsel-Hertsch et al., 2016). The functional link between G4 structures and processes important for cancer biology, including telomere maintenance, oncogene regulation, DNA replication and genome instability favours G4s as molecular targets for cancer therapies. Importantly, Hänsel-Hertsch et al. (2020) reported that increased G4 levels correlate with their sensitivity to G4-targeting by G4 ligands in breast cancer tumours, highlighting the potential of G4 DNA forming regions as a predictive biomarker for G4-ligand therapy (Hänsel-Hertsch et al., 2020).

Three different main therapeutic strategies have been investigated so far, which rely on changes in telomere maintenance, oncogene expression and increased genome instability.

In most types of cancer, telomerase activity is upregulated, which allows the cells to replicate without telomere shortening (Kim et al., 1994). Initial trials focused on

G4 stabilisation at telomeres to inhibit telomere extension by telomerase in cancer cells, thus preventing uncontrolled DNA replication leading to reduced tumour cell growth (Sun et al., 1997). G4 stabilisation in telomeres by G4-ligands has been shown to inhibit telomerase-mediated telomere extension by displacing components of the telomere protection complex shelterin (e.g. TRF2 and POT1) resulting in telomere DNA damage and apoptosis (Burger et al., 2005; Gowan et al., 2002; Phatak et al., 2007).

Another approach focuses on targeting G4-containing genes involved in cancer progression. Most promoters of oncogenes harbour G4 motifs (e.g. Myc, BCL2, VEGF, KRAS, KIT) and some of these promoter G4s have been associated with reduced gene expression (Cogoi and Xodo, 2006; Pandya et al., 2023; Siddiqui-Jain et al., 2002; Morgan et al., 2016). Hence, G4 stabilisation in promoters of oncogenes represents a potential target to reduce oncogenic gene expression in cancer cells, causing reduced tumour growth (Calabrese et al., 2018; Felsenstein et al., 2016; Hu et al., 2019). Early studies focused on modulating individual cancer genes by targeting their G4s (Bejugam et al., 2007; Cogoi and Xodo, 2006; Siddiqui-Jain et al., 2002). However, most G4 ligands bind multiple G4s and thus regulate the expression of many genes. Due to the prevalence of G4s in many cancer-promoting genes, targeting multiple G4s and thereby inhibiting the expression of many genes represents a feasible strategy to affect several pathways important for cancer progression. Global transcriptional profiling revealed that treatment of human pancreatic ductal adenocarcinoma (PDAC) cell lines with the G4 ligand CM03 induces global downregulation of many G4-containing genes involved in pathways important for PDAC progression. Moreover, this observation was associated with CM03-reduced tumour growth in PDAC xenografts (Marchetti et al., 2018). Nevertheless, it needs to be considered that particular G4 ligands may result in distinct profiles of affected genes involved in several pathways. Consequently, a better understanding of the pathways affected by G4 ligands could provide further insights into possible therapeutic opportunities.

“Misregulated” G4s, which form at the “wrong” time and place in the cell can lead to alterations within DNA replication, causing DNA damage-induced genome instability (Bryan, 2019). The capacity of G4 ligands to specifically induce genome instability, resulting in apoptosis of tumour cells, therefore provides another potential therapeutic approach. Particularly, cancer cells impaired in repair pathways such as homologous recombination (HR) and non-homologous end-joining (NHEJ) are sensitive to G4 ligands (McLuckie et al., 2013; Salvati et al., 2010; Zimmer et al., 2016). Currently, the G4 ligand CX-5461 is tested in human clinical trials for breast cancer patients with BRCA1/2 aberrations (Xu et al., 2017). Furthermore, G4 ligands used in combination with inhibitors of prominent DNA repair proteins revealed a synergistic effect on tumour growth (McLuckie et al., 2013; Wang et al., 2019b).

Altogether, G4 ligands present a potential therapeutic tool for anti-cancer immunotherapies. However, an important limitation is that G4-stabilising compounds such as pyridostatin (PDS) target all accessible G4s genome-wide. Therefore, future studies should consider rigorous control experiments using matched inactive compounds to distinguish direct from off-target effects. Furthermore, it remains essential to investigate which other cellular pathways are affected by their usage to minimise side effects and to optimise the therapeutic potential.

So far, a few G4 ligands have entered preclinical evaluation and early phases of clinical trials. However, none of them has progressed beyond phase II clinical trials. Some ligands, such as Braco-19, RHPS4, quarfloxin and APTO-253 have been withdrawn due to lack of efficacy, poor selectivity or cytotoxicity (Iachettini et al., 2013; Lim et al., 2009; Ohanian et al., 2021; Taetz et al., 2006). To date, only two G4 ligands are under investigation in clinical trials, including CX-5461 (phase Ib) and QN-302 (phase Ia) (Ahmed et al., 2023; Hilton et al., 2022). Facing the limitations of the already existing G4 ligands in clinical use, optimisation of the drug developmental process may lead to enhanced success rates of future G4 ligands.

1.5 Aims of the thesis

The evolutionarily conserved localisation of G4 motifs within the genome suggests that G4s play an important role in the regulation of biological processes. Their formation is cell type-specific and G4 structures are known to be dynamically regulated by G4-interacting proteins, cell cycle stages and exogenous stimuli. It is yet not completely understood when and how G4 structures form in living cells and which relevance they have for cellular functions and pathways. The functional role of G4 structures in cellular pathways has already been explored in several cell types. However, G4s have not been investigated in dendritic cells, so far. DCs are considered the most crucial type of APC that play a critical role in the induction of antigen-specific adaptive immunity and maintenance of immune homeostasis, thus presenting a potential target for therapeutic strategies in various diseases. Here, we hypothesise that G4 formation could assist specific targeting and regulation of DC function by modulating cellular pathways. The overarching aim of this thesis was therefore to investigate G4 formation and their role in cellular pathways in DCs by gaining insights into how G4 stabilisation affects DC phenotype and function and exploring the underlying mechanisms of G4-mediated cellular changes. These findings will broaden the knowledge of how DCs can be regulated on the genomic level to improve current therapeutic strategies. The specific aims of this study can be summarised as follows:

1. Characterisation of G-Quadruplex stabilisation on dendritic cell phenotype and function
2. Characterisation of G-Quadruplex stabilisation in dendritic cells as immunotherapeutic target in conditions of both, tumour (B16OVA melanoma) and GvHD
3. Investigation of the mechanism underlying G4-mediated expression of PD-L1, a major regulator of immunity, in dendritic cells

2. Materials and Methods

2.1 Materials

2.1.1 Equipment

Device	Company
BC-5000 Vet	Shenzhen Mindray Animal Medical Technology Co., Ltd.
BD FACSCanto II Flow cytometer	BD Biosciences
BD LSRFortessa Flow cytometer	BD Biosciences
BD FACS Aria III Cell sorter	BD Biosciences
Bioruptor [®] Pico	Diagenode SA
CFX96 [™] Real-Time PCR System	BioRad Laboratories Inc.
Chemidoc MP Imaging System	BioRad Laboratories Inc.
Chirascan VX spectrometer	Applied Photophysics Ltd.
FLUOstar Optima	BMG Labtech
ID7000 [™] 5L Spectral Cell Analyzer	Sony Biotechnology Inc.
MultiRad225	Prescision X-ray Inc.
4D-Nucleofector [®] (Core + X Unit)	Lonza Group AG
QuadroMACS Separator	Miltenyi Biotec
Thermo Scientific [™] Fluoroskan [™] FL Microplate Fluorometer and Luminometer	Thermo Fisher Scientific Inc.

ZEISS Axio Observer Z1 Microscope Carl Zeiss Microscopy GmbH

2.1.2 Consumables

Item	Company
Anti-Flag M2 Magnetic Beads	Sigma-Aldrich by Merck KGaA
Calibrite™ APC Beads	BD Biosciences
Compensation beads	BioLegend
Costar Transwell Permeable supports, 6.5 mm Insert, 5 µm polycarbonate membrane	Corning Inc.
Dynabeads™ MyOne™ Strepavidin C1	Invitrogen by Thermo Fisher Scientific Inc.
LS Columns	Miltenyi Biotec
Microscope cover glasses 14 mm Ø	Paul Marienfeld GmbH & Co. KG
Nitrocellulose Membrane	Amersham Biosciences

2.1.3 Chemicals & Reagents

Item	Company
2-Propanol	Carl Roth GmbH + Co. KG
Acetic Acid	Carl Roth GmbH + Co. KG
alamarBlue™ Cell Viability Reagent	Thermo Fisher Scientific Inc.

Ammonium persulfate (APS)	AppliChem GmbH
Ampicillin	Carl Roth GmbH + Co. KG
Ampuwa®	Fresenius Kabi GmbH
Benzonase	Sigma-Aldrich by Merck KGaA
Bovine serum albumin (BSA)	PanReac AppliChem GmbH
β-Mercaptoethanol	Gibco, Thermo Fisher Scientific Inc.
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen by Thermo Fisher Scientific Inc.
Collagenase from clostridium histolyticum 10 mg/mL	Sigma-Aldrich by Merck KGaA
CpG 1668 Oligonucleotide	TIB MOLBIOL
cOmplete protease inhibitor, tablet	Roche Holding GmbH
DAPI (4',6-diamidino-2-phenylindole)	Thermo Fisher Scientific Inc.
DAPI mounting media	Abcam Ltd.
Deoxyribonuclease I Typ IV from bovine pancreas, 750U (1 mg / mL)	Sigma-Aldrich by Merck KGaA
Dimethyl sulfoxide (DMSO)	PanReac AppliChem GmbH
Dulbecco's Modified Eagle Medium (DMEM), high glucose	Gibco by Thermo Fisher Scientific Inc.
Ethylenediaminetetraacetic acid (EDTA)	PanReac AppliChem GmbH

Fetal bovine serum (FBS)	Gibco by Thermo Fisher Scientific Inc.
Hoechst 33342	Molecular Probes by Thermo Fisher Scientific Inc.
Hydroxyurea	Merck KGaA
iQ™ SYBR® Green Supermix	BioRad Laboratories Inc.
Iscove's Modified Dulbeccos Medium (IMDM)	Gibco by Thermo Fisher Scientific Inc.
Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific Inc.
Lipopolysaccharide (LPS)	InvivoGen
Methanol 100 %	Carl Roth GmbH + Co. KG
Milk powder	Carl Roth GmbH + Co. KG
Mouse FLT-3 Ligand (FLT3L) Recombinant Protein	PeproTech® by Thermo Fisher Scientific Inc.
Opti-MEM™ (Reduced Serum Medium)	Gibco by Thermo Fisher Scientific Inc.
Ovalbumin	Sigma-Aldrich by Merck KGaA
Pageruler Plus Prestained Protein Standard	Thermo Fisher Scientific Inc.
Paraformaldehyde (PFA)	Sigma-Aldrich by Merck KGaA
PBS tablets	Gibco by Thermo Fisher Scientific Inc.
Penicillin/Streptomycin (Pen/Strep)	Gibco by Thermo Fisher Scientific Inc.

PhosSTOP phosphatase inhibitor, tablet	Roche Holding GmbH
Phusion® High-Fidelity PCR Master Mix	Thermo Fisher Scientific Inc.
Poly-D-Lysine	Gibco by Thermo Fisher Scientific Inc.
Proteinase K	QIAGEN
Recombinant human Interleukin-4 (rh IL-4)	BioLegend
Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF)	BioLegend
Recombinant Mouse CCL19	BioLegend
RNase A	PanReac AppliChem GmbH
Roswell Park Memorial Institute (RPMI) Medium 1640, GlutaMAX™ Supplement, HEPES	Gibco by Thermo Fisher Scientific Inc.
Rotiphorese® Gel 30 (37,5:1)	Carl Roth GmbH + Co. KG
SIINFEKL 1 mg/mL	Biotrend Chemikalien GmbH
Sodium dodecyl sulfate (SDS)	Thermo Fisher Scientific Inc.
STAT3 inhibitor (S3I-201)	Selleck Chemicals
TEMED	Carl Roth GmbH + Co. KG
Tissue Tek® O.C.T Compound	Sakura Finetek

Triton™ X-100	Sigma-Aldrich by Merck KGaA
Trypan Blue Solution Cell Culture	Sigma Aldrich by Merck KGaA
Trypsin-EDTA (0.25 %)	Gibco by Thermo Fisher Scientific Inc.
Tween® 20	Carl Roth GmbH + Co. KG

2.1.4 Cell culture media

Complete RPMI	RPMI Medium 1640 GlutaMAX™ supplemented with 10 % FBS, 1 % Pen/Strep, 50 µM β-Mercaptoethanol
Complete DMEM	DMEM Medium 1640 GlutaMAX™ supplemented with 10 % FBS, 1 % Pen/Strep
Complete IMDM	IMDM GlutaMAX™ supplemented with 10 % FBS, 1 % Pen/Strep, 50 µM β- Mercaptoethanol
Digestion medium	RPMI Medium 1640 GlutaMAX™ + 1 mg/mL Collagenase + 1 mg/mL DNase
Freezing medium	90 % FBS + 10 % DMSO

2.1.5 Buffers & solutions

6 x SDS buffer	120 mM DTT, 300 mM Tris-HCl pH 6.8, 9 % SDS, 48 % Glycerol, 0.1 % Bromphenol blue
Annexin V Binding Buffer	BioLegend

2x Binding and Washing (B&W) Buffer	10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2M NaCl
Binding Buffer (EMSA)	10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 150 mM KCl, 5% Glycerol
Blocking buffer (IF)	PBS + 3 % BSA
Blocking buffer (WB)	TBST + 5 % BSA
Cross-linking solution (ChIP)	RPMI Medium 1640 GlutaMAX™ supplemented with 10 % FBS, 1 % (v/v) formaldehyde
Detection Buffer (EMSA)	0.1 M Tris-HCl, 0.1 M NaCl pH 9.5
Fixation Buffer (IF)	Methanol + Acetic Acid (3:1)
10 x G4 folding buffer	100 mM Tris pH 7.5, 1 M KCl
HEPES Buffer Solution 1M	Gibco by Thermo Fisher Scientific Inc.
High-salt extraction buffer (EMSA)	20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM MgCl ₂ , 0.2 mM EDTA, 25 % glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A, 2 ng/mL leupeptin
Hypertonic Buffer (ChIP)	Chromatrap, Porvair Sciences Ltd.
Hypo-osmotic lysis buffer (EMSA)	0.3 M sucrose, 2 % (v/v) Tween 40, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl ₂ , 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A, 2 ng/mL leupeptin
Low-salt wash buffer (EMSA)	10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl ₂ , 0.1 mM EDTA, 0.5 mM DTT,

	0.5 mM PMSF, 2 ng/mL pepstatin A, 2 ng/mL leupeptin
Lysis Buffer (ChIP)	Chromatrap, Porvair Sciences Ltd.
Lysis Buffer (WB)	1x RIPA, Phosphatase inhibitor (1:100), Protease inhibitor (1:100)
MACS Buffer	0.5 % BSA , 2 mM EDTA in PBS
Maleic acid buffer (EMSA)	0.1 M maleic acid, 0.15 M NaCl pH 7.5
5x Native Loading dye	60 mM KCl, 10 mM Tris pH 7.5, 50 % (w/v) Glycerol, 0.025 % (w/v) Xylene cyanol, 0.025 % (w/v) Bromphenol blue
Pancoll human	PAN-Biotech GmbH
Permeabilisation Buffer (IF)	0.25 % (v/v) Triton X-100 in PBS
Red blood cell (RBC) lysis buffer	7.79 % (w/v) NH ₄ Cl, 0.035 % (w/v) EDTA disodium, 1 % (w/v) NaHCO ₃ , in ultra-pure water, pH 7.3
Resuspension buffer (pulldown)	25 mM Tris-HCl pH 7.4, 150 mM KCl, 2 mM MgCl ₂ , 1 mM DTT, 0.5 % IGEPAL, 1 mM PMSF, 1 x protease inhibitor
RIPA Buffer	Merck KGaA
20 x MOPS SDS running buffer	Invitrogen by Thermo Fisher Scientific Inc.
1.5 M sucrose buffer (EMSA)	1.5 M sucrose, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl ₂ , 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A, 2 ng/mL leupeptin

10 x TBE (Tris/Borate/EDTA)	108 g Tris base, 55 g boric acid, 40 mL 0.5 M EDTA (pH 8.0) in 1 L ddH ₂ O
Tris-buffered saline (TBS)	10 mM Tris-HCl (pH 7.5), 150 mM NaCl in H ₂ O
TBS + Tween 20 (TBST)	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 % Tween 20 in H ₂ O
Wash buffer (EMSA)	0.1 M maleic acid, 0.15 M NaCl pH 7.5, 0.3 % (v/v) Tween 20
Wash buffer (IF)	0.1 % (v/v) Tween 20 in PBS
Wash buffer (pulldown)	25 mM Tris-HCl (pH 7.4), 150 mM KCl, 2 mM MgCl ₂ , 1 mM DTT, 0.25% IGEPAL, 1 mM PMSF, 1 x protease inhibitor
X-Vivo20 serum free medium	Lonza Group AG

2.1.6 G4 ligands

Name	Catalogue number	Company
Pyridostatin	S7444	Selleck Chemicals
Braco-19	SML0560	Sigma-Aldrich by Merck KGaA
TMPyP4	613560	Sigma-Aldrich by Merck KGaA
CX-5461	5.09265	Sigma-Aldrich by Merck KGaA

2.1.7 Kits

Kit	Company
ArC™ Amine reactive compensation bead kit	Invitrogen by Thermo Fisher Scientific Inc.
DIG Luminescent detection kit	Roche Holding GmbH
DIG Oligonucleotide 3' End Labeling Kit 2 nd Generation	Roche Holding GmbH
Dual-Glo® Luciferase Assay System	Promega Corporation
FavorPrep™ Plasmid DNA Extraction Mini Kit	FAVORGEN Biotech Corp.
LEGENDplex™ Mouse Inflammation Panel (13-plex) with V-bottom Plate	BioLegend
MinElute Reaction Cleanup kit	QIAGEN
Mojosort™ CD3 selection kit	BioLegend
Mojosort™ CD4 naive T cell isolation kit	BioLegend
Mojosort™ CD8 naive T cell isolation kit	BioLegend
Mojosort™ CD90.2 selection kit	BioLegend
Mojosort™ Pan DC isolation kit	BioLegend
P3 Primary Cell 4D-Nucleofector™ X Kit S	Lonza Group AG

Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific Inc.
Qiaquick PCR purification kit for PCR cleanup	QIAGEN
Quick-RNA Miniprep Kit	Zymo Research Europe GmbH
RNeasy Mini Elute Kit	QIAGEN

2.1.8 Antibodies

2.1.8.1 FACS antibodies

Marker	Fluorochrome	Clone	Dilution	Cat. Number	Company
Annexin A5	FITC		1:50	640945	BioLegend
B220	BV510	RA3-6B2	1:200	103248	BioLegend
B220	BUV563	RA3-6B2	1:200	748868	BD Biosciences
CD11b	PE-Cy7	M1/70	1:400	101216	BioLegend
CD11b	BV650	M1/70	1:800	101259	BioLegend
CD11c	FITC	N418	1:400	117306	BioLegend
CD11c	PE-Dazzle	N418	1:400	117348	BioLegend
XCR1	PE	ZET	1:200	148204	BioLegend
MHC II (I-A/I-E)	PerCP-Cy5.5	AF6-120.1	1:400	116416	BioLegend

MHC II (I-A/I-E)	BUV496	M5/114.15.2	1:800	750281	BD Biosciences
CD4	PE	RM4-5	1:400	100512	BioLegend
CD8 α	PE-Cy7	53-6.7	1:400	100722	BioLegend
CD8 α	PE	53-6.7	1:400	100708	BioLegend
CD25	APC	PC61	1:200	102012	BioLegend
CD40	APC	3/23	1:200	124612	BioLegend
CD40	PE	3/23	1:200	124610	BioLegend
CD45	BV750	30-F11	1:400	103157	BioLegend
CD62L	APC	MEL-14	1:200	104412	BioLegend
CD62L	PerCP-Cy5.5	MEL-14	1:200	104432	BioLegend
CD69	BV421	H1.2F3	1:200	104528	BioLegend
CD80	BV421	16-10A1	1:200	104726	BioLegend
CD80	FITC	16-10A1	1:200	104706	BioLegend
CD86	BV421	GL-1	1:200	105032	BioLegend
PD-L1 (CD274)	APC	10F.9G2	1:200	124312	BioLegend
Zombie NIR™	NIR		1:1000	423106	BioLegend
CCR7 (CD197)	APC	4B12	1:100	120108	BioLegend

Tru Stain FcX	S17011E	1:100	101320	BioLegend
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2.1.8.2 *Western blot antibodies*

a. Primary antibodies

Specificity	Clone	Host species	Dilution	Cat. Number	Company
PD-L1		Goat	1:1000	AF1019	R&D Systems GmbH
IRF1	D5E4	Rabbit	1:1000	8478	Cell Signaling Technology Inc.
STAT3	C-20	Rabbit	1:1000	sc-482	Santa Cruz Biotechnology Inc.
Phospho-STAT3 (Tyr705)	3E2	Mouse	1:1000	9138	Cell Signaling Technology Inc.
Actin	C4	Mouse	1:2500	ab14128	Abcam Ltd.
GAPDH	7B	Mouse	1:2500	sc69778	Santa Cruz Biotechnology Inc.
Phospho-Histone H2A.X (Ser139) (γH2AX)	JBW301	Mouse	1:1000	05-636	Merck KGaA

b. Secondary antibodies

Specificity	Dilution	Cat. number	Company
IRDye® 680RD Donkey anti-goat IgG (H+L)	1:10,000	926-68074	LI-COR Biotech
IRDye® 800RD Donkey anti-mouse IgG (H+L)	1:10,000	926-32212	LI-COR Biotech
IRDye® 680RD Donkey anti-rabbit IgG (H+L)	1:10,000	926-68073	LI-COR Biotech

2.1.8.3 Immunofluorescence antibodies

Specificity	Dilution	Cat. Number	Company
BG4	0.5 ug		Self-made
DYKDDDDK Tag (D6W5B) (rabbit)	1:800	14793s	Cell Signaling Technology
Alexa Fluor™ 488 (anti-rabbit 2 nd Ab)	1:1000	A-11008	Invitrogen, Thermo Fisher Scientific Inc.
Cyanine 3 (anti-rabbit 2 nd Ab)	1:1000	A10520	Invitrogen by Thermo Fisher Scientific Inc.

2.1.9 Cell lines and primary cells

2.1.9.1 Cell lines

Cell line	Description
MuTu1940	Murine DC line originating from splenic tumours in CD11c:SV40LgT-transgenic C57BL/6 mice. Cells were a gift from Hans Acha-Orbea (Steiner et al., 2008)
HeLa	Human adenocarcinoma cell line, purchased from ATCC
B16OVA	Murine melanoma cell line that expresses the protein OVALBUMIN. Cells were a gift from Dominik Wolf

2.1.9.2 Primary cells

Cell type	Description
WT and IRF1 ^{-/-} Bone marrow-derived dendritic cells (BMDCs)	Stem cells isolated from tibia and femur of WT or IRF1 ^{-/-} C57BL/6J or 6N mice differentiated by FLT3-L. Bone marrow of IRF1 ^{-/-} mice was kindly provided by Sammy Bedoui (Peter Doherty Institute, Melbourne)
Human monocyte-derived dendritic cells (MoDCs)	Peripheral blood mononuclear cells from healthy donors differentiated by IL-4 and GM-CSF, Blood bank University Hospital Bonn, Germany
OT-I T cells	MHC class I-restricted, OVA ₂₅₇₋₂₆₄ -specific CD8 ⁺ T cells
OT-II T cells	MHC class II-restricted, OVA ₃₂₃₋₃₃₉ -specific CD4 ⁺ T cells

2.1.10 Mouse strains

Mouse Strain	Description
C57BL/6J and C57BL/6N	Wild-type strains (H-2K ^b), purchased from Janvier or Jackson Laboratories
Balb/c	Wild-type strain (H-2K ^d), purchased from Charles River
OT-I	C57BL/6J mice containing transgenic insert for Tcra-V2 and Tcrb-V5 genes. The transgenic T cell receptor specifically recognises ovalbumin peptide residues 257-264 (OVA ₂₅₇₋₂₆₄) in the context of H2-K ^b (CD8 co-receptor interaction with MHC class I). Mice were originally purchased from Taconic Biosciences.
OT-II	C57BL/6J mice expressing a transgenic T cell receptor that specifically recognises ovalbumin peptide residues 323-339 (OVA ₃₂₃₋₃₃₉) in the context of I-A ^b (CD4 co-receptor interaction with MHC class II). Mice were originally purchased from Taconic Biosciences.
C57BL/6N Cas9	Originally purchased from Jackson Laboratories (Stock No: 028551), modified at Walter and Eliza Hall Institute (Melbourne) to constitutively express Cas9

2.1.11 Software

Software	Company
BioRender	BioRender
FACSDiva™	BD Biosciences
Fiji (ImageJ)	NIH
FlowJo 10.10.0	BD Biosciences

Galaxy 24.1.3	Open-source, web-based platform for data-intensive biomedical research
GraphPad Prism 10	GraphPad Software Inc.
ID7000	Sony Biotechnology Inc.
IGV viewer 2.18.2	UC San Diego
Legendplex Data Analysis Software (v2024-06-15)	BioLegend
Microsoft Office Professional Plus 2019	Microsoft Corporation
Quantity One 1-D	BioRad Laboratories Inc.
ZEN Lite	Carl Zeiss Microscopy GmbH
Zotero 6.0.36	Open-source software for references

2.2 Methods

2.2.1 Animals

C57BL/6 and BALB/c mice were housed in specific pathogen-free conditions at the immunological Facility for Experimental Therapy, University Hospital Bonn or the Melbourne Bioresources Platform at Bio21 Molecular Sciences and Biotechnology Institute. Rag2-deficient OT-I TCR transgenic mice (Rag2/OT-I, Taconic Biosciences) and Rag2-deficient OT-II TCR transgenic mice (Rag2/OT-II, Taconic Biosciences) were maintained by intercrossing in the house of experimental therapy, University Hospital Bonn. All experiments were approved by the local authorities of North Rhine Westphalia under the license 81-04.02.2020.A401 or by the Animal Ethics Committee of the University of Melbourne (Ethics Application: 21410). Mice were used at 8 – 12 weeks of age

and housed in our facilities for at least one week after purchase prior to experiments.

2.2.2 Cell culture

2.2.2.1 *MuTu 1940*

The murine DC line MuTu1940 was cultured in IMDM GlutaMAX™ supplemented with 10 % FBS, 1 % Pen/Strep, 25 mM HEPES and 50 µM β-Mercaptoethanol in a humidified incubator at 37 °C and a 5 % CO₂ atmosphere. Cells were passaged at 70 % confluency using PBS supplemented with 5 mM EDTA.

2.2.2.2 *HeLa*

Human HeLa cells were grown in DMEM supplemented with 10 % FBS and 1 % Pen/Strep at 37 °C in 5 % CO₂. Cells were split twice per week using Trypsin-EDTA.

2.2.2.3 *B16OVA melanoma*

The mouse melanoma cell line B16OVA was cultured in RPMI 1640 GlutaMAX™ supplemented with 10 % FBS, 1 % Pen/Strep, 25 mM HEPES and 50 µM β-Mercaptoethanol at 37 °C and 5 % CO₂. The medium was exchanged every second day. Cells were passaged once per week using Trypsin-EDTA.

2.2.3 Generation of murine bone marrow-derived dendritic cells

Tibia and femur of C57BL/6 mice were cut at one end and bone marrow was isolated by centrifugation (5,000 g, 30 sec). Following red blood cell (RBC) lysis, stem cells were filtered through a 70 µm cell strainer and washed twice with PBS. Afterwards, cells were counted, resuspended in complete RPMI 1640 GlutaMAX™ supplemented with 200 ng/mL murine-recombinant FLT3L (PeproTech) and seeded at a concentration of 1.5×10^6 cells/mL in 6-well or 12-well plates for differentiation. Cells were cultured in a humidified atmosphere at 37 °C and 5 % CO₂ for 8 days without replating. On day 7, differentiated immature

bone marrow-derived DCs (BMDCs) were treated with the respective stimuli (section 2.2.6) for 24 h. BMDCs are non-adherent and were harvested through gently pipetting up and down.

2.2.4 Generation of human monocyte-derived dendritic cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors' buffy coats, kindly provided by the blood bank of the University Hospital Bonn, by Ficoll-Paque density gradient according to standard procedures. Briefly, blood was diluted 1:2 with PBS. Diluted blood was carefully layered on top of the Ficoll (Pancoll human, PAN-Biotech) and cells were isolated by density gradient separation for 15 min at 1800 rpm (break switched off). The milky cell layer (composed of monocytes and lymphocytes) was transferred to a new Falcon tube and washed twice with PBS. Afterwards, cells were counted, resuspended at a concentration of 1×10^7 cells/mL in XVivo20 medium and incubated at 37 °C in a humidified atmosphere at 5 % CO₂. Cells were allowed to adhere to polystyrene flasks for up to 90-120 min for monocyte enrichment. Non-adherent cells were removed by rinsing the flasks 2-3 times with PBS. Enriched monocytes were incubated in RPMI 1640 GlutaMAX™ supplemented with 10 % FBS, 2 % Pen/Strep, 100 ng/mL human rGM-CSF and 20 ng/mL rIL-4 for 7 days. Cytokines were added every second day. On day 6, immature monocyte-derived DCs (MoDCs) were treated with 10 µM PDS or 100 ng/mL LPS for 24 h. Afterwards, cells were harvested via detaching with PBS + 5 mM EDTA and immediately used for further experiments.

2.2.5 Cell viability assay

Cells were seeded at a concentration of 2×10^4 cells in 100 µl medium in 96-well plates. The next day, cells were treated with a range of concentrations of the respective reagent (PDS, Braco-19, CX-5461, TMPyP4 and hydroxyurea (HU)) for 24 h. Afterwards, the medium was aspirated and replaced by fresh medium supplemented with AlamarBlue in a 1:10 dilution. Following an additional 4 h of

incubation at 37 °C, fluorescence (560/590nm) was measured using a multiplate reader. Samples were measured in technical triplicates. Fluorescent intensity was normalised to untreated cells (negative control). A cell viability threshold $\geq 80\%$ was used to decide on an appropriate compound concentration to avoid possible effects based on apoptotic or necrotic mechanisms.

2.2.6 DC stimulation *in vitro*

MuTu DC1940 cells were stimulated with 0.5 μM PDS for 24 h prior to experimental use.

Immature MoDCs were stimulated with 10 μM PDS for 24 h or 100 ng/mL LPS (DC activation control) for 16 h on day 6 of differentiation.

Ex vivo cultured immature BMDCs were treated on day 7 of differentiation with 10 μM PDS, 20 μM Braco-19, 40 nM CX-5461, 20 μM TMPyP4, 100 μM HU or 1 mM CpG (DC activation control) for 24 h. For STAT3 inhibition, BMDCs were treated with 25 μM of the STAT3 inhibitor S3I-201, 6 h prior to PDS addition.

Depending on the reagent's solvent, sterile filtered H₂O or DMSO was used as vehicle control. All cells were cultured in a humidified atmosphere at 37 °C in 5 % CO₂.

2.2.7 B16OVA tumour models

B16OVA cells were detached using Trypsin-EDTA and washed twice with PBS. For *in vivo* use, cells were counted and resuspended at a concentration of 10×10^6 cells/mL in PBS.

2.2.7.1 B16OVA melanoma

5×10^5 B16OVA cells/mouse were injected subcutaneously (s.c.) into the shaved flank of C57BL/6 mice. Once the tumour reached a size $> 40 \text{ mm}^3$, 0.5 mg PDS dissolved in PBS or 50 μl PBS (vehicle control) were injected intratumourally (i.t.).

Two days after intratumoural injection, mice were euthanised and tumours and tumour-draining lymph nodes (inguinal and iliac) were harvested for further experimental analysis. Mouse well-being and weight were daily monitored and tumour size was measured every second day.

2.2.7.2 B16OVA metastasis

5×10^5 B16OVA cells/mouse were injected intravenously (i.v.) via the tail vein to induce lung metastasis. Three days prior to B16OVA cell injection, administration of PDS (7 mg/kg) i.v. was started. PDS was injected every second day. On day 14 after the B16OVA injection, mice were euthanised and lungs were harvested for further experimental use.

2.2.8 Acute Graft-versus-Host-Disease model

In the MHC-mismatched aGvHD model, C57BL/6 mice (H-2K^b) served as transplant donors and BALB/c (H-2K^d) as recipients. One day prior to transplantation (day -1), Balb/c mice were subjected to total body irradiation (TBI) with two doses of 2.5 Gy (MultiRad225, Precision X-Ray), with a 4 h recovery phase in between. 24 h later (day 0), bones (tibia and femur) and spleens of sex- and age-matched C57BL/6N mice were collected for BM and T cell isolation. BM was harvested by centrifugation (10,000 g, 15 min). The cell pellet was resuspended in MACS Buffer and filtered through a 70 µm nylon mesh. BM cells were depleted of CD90.2⁺ cells using the Mojosort™ CD90.2 selection kit (BioLegend). In parallel, the spleen was mashed, filtered and T cells were isolated using the Mojosort™ CD3 selection kit (BioLegend). 5×10^6 T-cell depleted BM cells were mixed with 6×10^5 CD3⁺ T cells in PBS and injected i.v. via the tail vein into BALB/c mice.

Two days prior to transplantation (day -2), PDS treatment was started. 7 mg/kg PDS dissolved in Ampuwa was administered intraperitoneally (i.p.) every other day.

Mice were daily monitored and scored for weight loss, skin, fur, activity and posture according to Riesner et al. (2016) for up to 60 days. At indicated time points, blood was drawn from the heart and organs (skin, spleen, liver, small intestine) were harvested for histological or flow cytometric analysis.

2.2.9 Quantification of haematological parameters

Mice were euthanised by CO₂ and blood was extracted from the heart and collected in EDTA tubes on day 14 after transplantation. Haematological parameters were immediately quantified using the haematology analyser BC-5000 Vet (Shenzhen Mindray Animal Medical Technology Co., Ltd.).

2.2.10 Immunofluorescence staining of G4s

Glass coverslips were coated with 50 µg/mL Poly-D-Lysine (Gibco) for 1 h at room temperature (RT). Cells were seeded on coated coverslips in a 24-well plate at a concentration of 2×10^5 cells/mL. The next day, cells were treated with G4 ligands (PDS, Braco-19, CX-5461, TMPyP₄) at indicated concentrations for 24 h. Cells were prefixed with 50 % complete RPMI medium and 50 % ice-cold methanol/acetic acid (3:1) at 4 °C for 5 min followed by fixation with ice-cold methanol/acetic acid (3:1) for 10 min at 4 °C. Cells were then permeabilised with 0.25 % (v/v) Triton X-100 in PBS for 10 min at RT and incubated with blocking solution (3 % bovine serum albumin (BSA) in PBS) for 1 h at RT. Next, cells were incubated with 0.5 µg of the G4-specific single-chain antibody BG4 (purified by ourselves as previously described (De Magis et al 2020)) in blocking solution for 2 h at RT followed by incubation with an anti-Flag antibody against the DYKDDDDK epitope (Cell Signalling) diluted 1:800 in blocking solution for 1 h at RT. Afterwards, cells were incubated with Cyanine 3 goat-anti-rabbit IgG (Invitrogen) diluted 1:1000 in blocking solution for 1 h at RT. Following each incubation step, cells were washed three times with 0.1 % (v/v) Tween 20 in PBS for 10 min at RT under gentle agitation. The coverslips were mounted using Fluoroshield mounting media (Abcam) containing DAPI (4',6-diamidino-2-

phenylindole) for nuclear staining and dried overnight. Slides were visualised using the Zeiss Axio Observer Z1 fluorescence microscope. Fluorescence signal intensity was determined using Fiji (ImageJ).

2.2.11 Western blot

5×10^5 cells were lysed in 1 x RIPA buffer supplemented with 1 x protease and phosphatase inhibitor and incubated on ice for 30 min. Afterwards, cells were centrifuged at 13,000 g and 4 °C for 10 min. Protein-containing supernatant was mixed with 6 x SDS buffer and denatured at 95 °C for 5 min. Alternatively, cells were lysed in 1x SDS buffer supplemented with 2.5 U/μl Benzonase (Sigma-Aldrich) for 10 min at 37 °C before denaturing. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10-15 % polyacrylamide gels for 1.5 h at 100 V and blotted onto a nitrocellulose membrane (GE Healthcare) with 400 mA for 1 h. The membrane was blocked with 5 % BSA in TBS-T for 1 h to saturate free binding sites. Afterwards, the membrane was incubated with the appropriate primary antibodies (Section 2.1.8.2 a)) in blocking solution at 4 °C overnight. The next day, the membrane was washed three times with 1 x TBST for 10 min and incubated with the respective fluorescently labelled secondary antibody (Section 2.1.8.2 b)) in blocking buffer for 1 h at RT protected from light. Following another three washing steps with 1 x TBST, protein signals were detected using the ChemiDoc MP Imaging System (BioRad). Size standard was obtained using the PageRuler Prestained NIR Protein ladder (Thermo Fisher Scientific). Signal intensities were determined using the QuantityOne software (BioRad).

2.2.12 Flow cytometry

2.2.12.1 Surface molecule staining

In vitro-cultured cells were collected and immediately used for multicolour staining at a concentration of 1×10^6 cells/mL. Tissues and organs were harvested, mashed and digested in RPMI 1640 GlutaMAX medium supplemented with 1

mg/mL Collagenase A (Sigma-Aldrich) and 100 µg/mL DNase I (Sigma-Aldrich) for 30 min at 37 °C. Digested tissues and organs were filtered through a 70 µm cell strainer and 1:10 of the cells were used for multicolour analysis. To distinguish live from dead, cells were stained with Zombie-NIR (BioLegend) diluted 1:1000 in MACS buffer (PBS + 0.5% BSA + 2 mM EDTA) for 15 min at RT in the dark. Afterwards, cells were stained for surface molecules with fluorochrome-conjugated antibodies (Section 1.1.8.1) diluted in MACS buffer for 20 min at 4 °C in the dark. Cells were washed twice with PBS, resuspended in MACS buffer and filtered. Single-cell suspensions were acquired on FACSCanto II Flow Cytometer (BD Biosciences) or LSR Fortessa (BD Biosciences) using the FACSDiva software (BD Biosciences) or ID 7000 Spectral Cell Analyzer (Sony Biotechnology) using the ID7000 system software. Data analysis was performed using FlowJo software (version 10.10.0) and Prism 10 (GraphPad).

Cell sorting was conducted by the Flow cytometry and imaging facility at the Murdoch Children's Research Institute (MCRI, Victoria, Australia) using the FACSria III Cell sorter (BD Biosciences).

2.2.12.2 Apoptosis assay

Cells were stained with Annexin V (BioLegend) and Zombie NIR (BioLegend) according to manufacturer's instructions. Briefly, cells were harvested, washed and resuspended in 1 x Annexin V binding buffer (BioLegend) at a concentration of 1×10^6 cells/mL. Cells were stained with Annexin V (1:50) and Zombie NIR (1:1000) diluted in 1 x Annexin V binding buffer for 15 min at RT in the dark. Cells were washed twice with PBS and resuspended in MACS buffer. The frequency of apoptotic cells was assessed within 1 h using the FACSCanto II Flow Cytometer (BD Biosciences).

2.2.12.3 Cell cycle analysis

Cells were harvested and fixed with 2 % PFA in PBS for 15 min at RT followed by permeabilisation with 0.25 % Triton X-100 in PBS for 5 min at RT. After two washing steps with PBS, cells were stained with 10 µg/mL DAPI and 50 µg/mL RNase A diluted in MACS buffer for 30 min at 37 °C. Then, cells were washed, resuspended in 500 µl MACS buffer and analysed within 1 h using the FACSCanto II Flow Cytometer (BD Biosciences) at low speed.

2.2.13 *In vitro* DC migration assay

DC migratory capacity was evaluated by a transwell assay using 24-well plates containing 6.5 mm transwell inserts with 5 µm pore polycarbonate membrane (Corning). BMDCs were harvested on day 8 of differentiation and resuspended in complete RPMI 1640 GlutaMAX medium at a concentration of 4×10^5 cells/mL. Complete RPMI medium supplemented with 100 ng/mL murine CCL19 (BioLegend) was placed at the bottom of each well. 2×10^5 cells were added to the top chamber and incubated at 37 °C for 3 h. Afterwards, migrated cells at the bottom of the wells were harvested, resuspended in MACS buffer and counted by flow cytometry using the FACSCanto II Flow Cytometer (BD Biosciences). The total amount of migrated cells was calculated using APC counting beads.

2.2.14 Dextran uptake assay

Differentiated BMDCs were seeded at a concentration of 2×10^5 cells in 100 µl complete RPMI medium in 96-flat-well plates and incubated at 37 °C for 2 h. FITC-Dextran (1 mg/mL) was added directly to the wells and cells were further incubated for 15, 30, 45, 60 and 90 min at 37 °C to facilitate Dextran uptake. Additional samples with FITC-Dextran were incubated for 90 min on ice in the dark (negative control). After incubation, cells were washed three times with ice-cold PBS, resuspended in MACS buffer and analysed by flow cytometry using the FACSCanto II Flow Cytometer (BD Biosciences). Antigen uptake was calculated by normalising $\text{MFI}_{\text{sample}}$ to MFI_{0min} .

2.2.15 Cytokine analysis

Supernatants from cultured cells were collected after 24 h of stimulation or 72 h of co-culture and were stored at -20 °C until usage. Cytokines were quantified using pre-defined panels (Table 1) of the flow cytometry-based multiplex immunoassay LEGENDplex™ (BioLegend), which allowed the analysis of several cytokines in one assay. After thawing, samples were processed according to manufacturer's instructions. Cell culture supernatant was diluted 1:1 in Assay buffer. Samples and Standards were run in technical duplicates. Data were acquired at the ID 7000 Spectral Cell Analyzer (Sony Biotechnology).

Table 1 | Pre-defined panels used for cytokine analysis

Mouse inflammation Panel	Mouse Th Cytokine Panel
IL-23, IL-1 α , IFN- γ , TNF- α , CCL2, IL-12p70, IL-1 β , IL-10, IL-6, IL-27, IL-17A, IFN- β , GM-CSF	IFN- γ , IL-5, TNF- α , IL-2, IL-6, IL-4, IL-10, IL-9, IL-17A, IL17F, IL-22, IL-13

2.2.16 *In vitro* T cell stimulation

Differentiated BMDCs (day 8) were pulsed with 2 μ g/mL OVA₂₅₇₋₂₆₄ (SIINFEKL, Biotrend) for 1 h or 100 μ g/mL OVA₃₂₃₋₃₃₉ (Sigma-Aldrich) for 4 h at 37 °C. Meanwhile, the spleen and lymph nodes of Rag2/OT-I and Rag2/OT-II mice were harvested, mashed and filtered through a 70 μ m cell strainer followed by RBC lysis and again filtering. CD4⁺ and CD8⁺ T cells were isolated by magnetic-activated cell separation (MACS) using the Mojosort Mouse CD4 or CD8 Naïve T Cell Isolation Kit (BioLegend) with MACS Columns (Miltenyi Biotec) according to manufacturer's instructions. To monitor proliferation, isolated T cells were stained with 5 μ M carboxyfluorescein succinimidyl ester (CFSE) for 12 min at 37 °C followed by immediate stopping of the reaction with complete RPMI medium. T cells were then washed and cultured with OVA peptide-loaded DCs at a concentration of 1 x 10⁵ cells in a 2:1 ratio for up to three or four days. T cell stimulation was determined by analysing T cell surface molecule expression after

24 h of co-culture, while T cell proliferation was assessed by CFSE signal reduction using the FACSCanto II Flow Cytometer (BD Biosciences).

2.2.17 RNA isolation and next-generation sequencing

On day 8 of differentiation, pan-DCs were isolated from bulk BMDCs using the Mojosort™ Pan DC isolation kit (BioLegend) according to manufacturer's instructions. Total RNA from pan-DCs was then extracted using the Quick-RNA Miniprep Kit (Zymo Research) following manufacturer's protocol. RNA concentration and purity were determined using the NanoDrop 1000 spectrophotometer. RNA quality control, QuantSeq 3'-mRNA library preparation, sample run (Illumina NovaSeq 6000, 100 bp single-end) and primary data analysis was carried out by the Next Generation Sequencing (NGS) Core Facility (University of Bonn). Data processing, including quality control, genome alignment, read count normalisation and differentially expressed gene (DEG) analysis was performed according to Batut et al. (2021) using Galaxy (version 24.1.3).

2.2.18 Thioflavin T staining

2.5 µg of the respective oligonucleotides (Table 2) were diluted in 1 x G4 folding buffer (10 mM Tris-HCl pH 7.5, 100 mM KCl) and incubated for 5 min at 95 °C to allow G4 structure formation. Samples were cooled down to RT for at least 30 min before further usage. Subsequently, 10 µl of samples were mixed with 2.5 µl 5 x Native loading dye and run on a 15 % TBE (Tris/Borate/EDTA) native gel for 90 min at 80 V. The gel was pre-run for 15 min at 80 V and the wells were thoroughly rinsed before sample loading. Afterwards, the gel was stained with 0.5 µM Thioflavin T (ThT) in 1 x G4 folding buffer for 15 min under agitation protected from light. ThT signal was detected using the Chemidoc MP Imaging system (BioRad). Next, the gel was incubated with 0.5 µg/mL ethidium bromide in 1 x TBE for 15 min in agitation to stain the total DNA. Ethidium bromide was detected using the Chemidoc MP Imaging system (BioRad).

Table 2 | Oligonucleotide sequences for ThT staining and CD.

Name	Sequence
PD-L1 G4 motif	GCGGAGTGGGCGGGGCTCTGAACTCGAGATAAGACCAGGAAATCGTG GTCCCAAGCCTCATGCCAGGCTGCACTTGACGTGCGGGGCCAGTC TCCTCGCCTGCAGGTAAGGGAG
Scrambled control	GCGTAGTGCGCGACGCTCTGAACTCGAGATCAGACGAGCAAGTCGTG ATCGCGAAGCGTCATGCAACGCTGCACGTGCACGTGCGGAGCACGTC TGCTCGCTCGCGAGTGAGCGAG
PD-L1 G4 mutant	GCGGAGTGCGCCGCGCTCTGAACTCGAGATAAGACCAGCAAATCGTG GTCCCAAGCCTCATGCCACGCTGCACTTGACGTGCGCGGCCAGTC TCCTCGCCTGCAGGTAAGCGAG

2.2.19 Circular dichroism

5 μ M of the respective oligonucleotides (Table 2) were diluted in 1 x G4 folding buffer. Samples were incubated at 95 °C for 5 min and slowly cooled down to RT overnight. Absorbance was acquired with a Chirascan VX spectrometer (Applied Photophysics) using the indicated settings (Table 3).

Table 3 | CD measurement settings

Measurement Range	200-350 nm
Data pitch	1 nm
Band width	2 nm
Sec response	0.5 sec
Sensitivity	Standard
Scanning speed	200 nm/min

2.2.20 G4 Chromatin immunoprecipitation

G4 chromatin immunoprecipitation (ChIP) was performed as previously described (Hänsel-Hertsch et al., 2018) with minor changes. 1×10^7 cells were resuspended in cross-linking solution (RPMI GlutaMAX™ supplemented with 10 % FBS and 1 % (v/v) formaldehyde) and incubated at RT for 10 min in agitation. The cross-linking reaction was stopped by adding 0.12 M glycine and incubating the mixture for 10 min in gentle agitation at RT. Nuclei were isolated by incubating cells for 10 min at 4 °C in Chromatrap hypotonic buffer followed by nuclei lysis in Chromatrap lysis buffer for 30 min on ice. DNA was sonicated in 16 cycles (30 sec on/ 30 sec off) using a Bioruptor® Pico (Diagenode). Due to low chromatin concentrations, the volume of G4 ChIP reactions was quadrupled to start with an optimal chromatin input concentration of 1 µg. Hence, G4 ChIP reactions containing 174 µl blocking buffer (25 mM HEPES pH 7.5, 10.5 mM NaCl, 110 mM KCl, 1 mM MgCl₂, 1 % (w/v) BSA), 4 µl RNase A and 10 µl chromatin (1 µg) were incubated at 37 °C and 1400 rpm for 20 min. 800 ng BG4 was added to the samples and incubated for 1 h at 16 °C under rotation. The following steps were conducted as described by Hänsel-Hertsch et al. (2018). G4 ChIP reactions were incubated with anti-FLAG M2 magnetic beads (Sigma-Aldrich) for 1 h at 16 °C. Afterwards, beads were washed three times with wash buffer (100 mM KCl, 0.1 % (v/v) Tween 20, 10 mM Tris-HCl pH 7.4). DNA was eluted from the beads by incubating samples in TE buffer supplemented with proteinase K in agitation for 1 h at 37 °C followed by 2 h at 65 °C. Eluted DNA was purified using the MinElute Reaction Cleanup kit (Qiagen) and G4 enrichment was assessed by quantitative polymerase chain reaction (qPCR).

2.2.21 Quantitative PCR

Quantitative PCR (qPCR) was performed using the iQ™ SYBR® Green Supermix (BioRad). The gene-specific primers used are listed in Table 4. Reactions were set up and qPCR was performed as indicated (Table 5 + 6) using the CFX96™ Real-Time PCR System (BioRad). Samples were run in technical duplicates.

Enrichment of the BG4 immunoprecipitated DNA region of interest was normalised over input.

Table 4 | Primer set for qPCR

Primer	Sequence
PD-L1 prom G4 fwd	AGGGACTGGGTGGCTTCGGTTT
PD-L1 prom G4 rev	AAGTGCCCTGCAAGCGGATTCC

Table 5 | qPCR Mastermix

Reagent	Volume per reaction
iQ™ SYBR® Green Supermix (2x)	6 µl
Primer fwd (1 µM)	1.25 µl
Primer rev (1 µM)	1.25 µl
Template	2.5 µl
Nuclease free water	1 µl
Total volume	12 µl

Table 6 | qPCR Program

Temperature	Time (min:sec)	Cycles
95 °C	3:00	1
95 °C	0:15	39
62 °C	0:15	
72 °C	0:30	

2.2.22 Restriction-free cloning

Target DNA sequences were inserted into the Luciferase reporter vector psiCHECK2™ (Promega) downstream of the HSV-TK promoter in front of the firefly luciferase gene using the restriction-free (RF) cloning method (Van Den Ent and Löwe, 2006). A megaprimer was used for RF cloning PCR, which consisted of the insert and 50 bp flanking regions (on the 5'- and 3' end) complementary to the plasmids desired cloning site. The megaprimer was assembled and extended in two rounds of PCR (PCR A, PCR B) as indicated below (Table 7-11) using the Phusion® High-Fidelity DNA polymerase (Thermo Fisher Scientific). Following the second PCR, the megaprimer was purified using the Qiaquick PCR purification kit (Qiagen) and the concentration was determined. The cloning PCR (PCR C) was conducted as indicated below (Table 12 + 13). Afterwards, the PCR reaction

was treated with 1 µl Dpn I for 2 h at 37 °C to digest the methylated parental plasmid. The cloned plasmid was transformed into chemically competent DH5α cells by heat shock. Successful insertion of the target sequence into the vector was verified by Sanger sequencing.

Table 7 | Primers for megaprimer assembly

Name	Sequence
PD-L1 G4 template	GGAGTGGGCGGGGCTCTGAACTCGAGATAAGACCAGGAAATCGTGGT CCCCAAGCCTCATGCCAGGCTGCACTTGACGTGCGGGGCCAGTCTC CTCGCCTGCAGGTAAGGG
G4 Primer 1 fwd	AGCGACCCTGCAGCGACCCGCTTAAGGAGTGGGCGGGGCTCTGAA
G4 Primer 2 rev	TACCAACAGTACCGGAATGCCAAGCTTCCCTTACCTGCAGGCGAGGA
Scrambled template	GTAGTGCGCGACGCTCTGAACTCGAGATCAGACGAGCAAGTCGTGATC GCGAAGCGTCATGCAACGCTGCACGTGCACGTGCGGAGCACGTCTGC TCGCTCGCGAGTGAGCG
Scramble Primer 1 fwd	AGCGACCCTGCAGCGACCCGCTTAAGTAGTGCGCGACGCTCTGAA
Scramble Primer 2 rev	TACCAACAGTACCGGAATGCCAAGCTTCGCTCACTCGCGAGCGAGCA-3'
Extension Primer 3 fwd	ACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAACACCGAGCGACCCT GCAGCGACCCGCTTAA
Extension Primer 4 rev	GGGCCCTTCTTAATGTTCTTAGCATCGGCCATGGTGGCTTTACCAACAGTA CCGGAATGCCAAGCTT

Sequence of Insert

Upstream plasmid sequence

Plasmid sequence upstream of cloning site

Downstream plasmid sequence

Plasmid sequence downstream of cloning site

Table 8 | Mastermix PCR A

Reagent	Volume per reaction
Phusion Mastermix (2x)	10 µl
Primer 1 fwd (10 µM)	1 µl
Primer 2 rev (10 µM)	1 µl
Template	1 µl
Nuclease free water	7 µl
Total volume	20 µl

Table 9 | Program PCR A

Temperature	Time (min:sec)	Cycles
95 °C	2:00	1
95 °C	0:20	44
54 °C	0:20	
72 °C	0:05	
72 °C	1:00	1

Table 10 | Mastermix PCR B

Reagent	Volume per reaction
Phusion Mastermix (2x)	10 µl
Primer 3 fwd (10 µM)	1 µl
Primer 4 rev (10 µM)	1 µl
Product PCR A	0.5 µl
Nuclease free water	7.5 µl
Total volume	20 µl

Table 11 | Program PCR B

Temperature	Time (min:sec)	Cycles
95 °C	2:00	1
95 °C	0:20	44
54 °C	0:20	
72 °C	0:04	
72 °C	1:00	1

Table 12 | Mastermix PCR C

Reagent	Volume per reaction
Phusion Mastermix (2x)	10 µl
Vector, 100 ng/µl	1 µl
Megaprimer, 100 ng/µl	0.5 µl
Nuclease free water	8.5 µl
Total volume	20 µl

Table 13 | Program PCR C

Temperature	Time (min:sec)	Cycles
98 °C	0:30	1
98 °C	0:08	19
72 °C	1 min/kb	
72 °C	5:00	1

2.2.23 Luciferase reporter assay

HeLa cells were seeded at a concentration of 1×10^5 cells per 12-well. The next day, HeLa cells were transfected with the psiCHECK™ 2 reporter plasmid containing the sequence of interest using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's protocol. Briefly, 1.6 µg DNA was diluted in 100 µl OptiMEM per sample. Furthermore, 100 µl OptiMEM was supplemented with 4 µl Lipofectamine 2000 and incubated for 5 min at RT. Afterwards, the diluted DNA was mixed with the diluted Lipofectamine 2000 and incubated for 20 min at RT prior to adding the DNA-Lipofectamine complexes to the cells. After 6 h of incubation, the medium was exchanged. 24 h following transfection, the medium was aspirated and cells were lysed in 200 µl lysis buffer (Promega) for

15 min at RT in agitation. 50 µl of cells were transferred to a 96-well plate and mixed with an equal amount of Luciferase Assay Reagent (Dual-Glo Luciferase buffer substituted with Dual-Glo Luciferase Substrate (1:50)). Following incubation for 10 min, firefly luminescence was measured using the Thermo Scientific™ Fluoroskan™ FL Microplate Fluorometer and Luminometer (Fisher Scientific). Afterwards, 50 µl of the Dual-Glo Stop & Glo Reagent (Dual-Glo Stop & Glo Buffer substituted with Dual-Glo Stop & Glo Substrate (1:50)) was added and samples were incubated for further 10 min before measuring Renilla luminescence. The ratio of Firefly to Renilla luminescence was calculated. Samples were measured in technical duplicates.

2.2.24 Generation of BMDC knockouts using the Alt-R CRISPR-Cas9 system

Stem cells were isolated from Cas9 mice as previously described (section 2.2.3). Target-specific Alt-R CRISPR RNA (crRNA) oligonucleotides (IDT) were produced based on the target sequences (Table 14). crRNA oligonucleotides and trans-activating crRNA (tracrRNA) oligonucleotides (IDT) were reconstituted to 100 µM in Nuclease-Free Duplex Buffer (IDT). The two oligonucleotides were mixed at equimolar concentrations to form the guide RNA (gRNA) duplex. Oligonucleotides were annealed at 95 °C for 5 min followed by hybridisation for 15 min at RT using a Thermocycler. The formed gRNA was transfected into Cas9 stem cells using nucleofection as previously described (Freund et al., 2020). Briefly, 2×10^6 cells per reaction were resuspended in 20 µl P3 primary nucleofection solution (16.4 µl P3 Primary cell Nucleofector Solution, 3.6 µl Supplement 1) (Lonza). 1 µl of the gRNA duplex was added per sample and mixed carefully. The cell/gRNA mix was then loaded into the nucleofector cassette strip and nucleofected using the Lonza 4D-Nucleofector (4D-Nucleofector Core Unit: Lonza, AAF-1002B; 4D-Nucleofector X Unit: AAF-1002X) with the Buffer P3, CM-137 condition. Immediately after nucleofection, 180 µl complete RPMI 1640 GlutaMAX™ supplemented with 200 ng/mL FLT3L was added to each cassette well. Cells were transferred to a 24-well plate and cultured

as described above for 7 days to differentiate into BMDCs. KO efficiency was analysed by flow cytometry.

Table 14 | cRNA target sequences

Gene	gRNA Target sequence
STAT3 # 1	ACAGGCAUCGGGCCACGAUC
STAT3 # 2	TCACTACGGCGGCTGTTGGG
PD-L1	TGCTGCATAATCAGCTACGG

2.2.25 Pulldown assay

2.5 µg of 5'-end biotinylated oligonucleotides (IDT) (Table 15) were folded in G4 folding buffer for 5 min at 95 °C, followed by cooling down to RT for 30 min. Biotinylated oligonucleotides were coupled to Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen) according to manufacturer's instructions. Briefly, beads were washed and resuspended in 2 x Binding and Washing (B&W) Buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl). Biotinylated DNA oligonucleotides were added in an equal volume to the beads and incubated for 1 h at RT in gentle rotation. Coated beads were washed 2-3 times with 1 x B&W buffer, resuspended in resuspension buffer (25 mM Tris-HCl pH 7.4, 150 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.5 % IGEPAL, 1 mM PMSF, 1 x protease inhibitor) and stored on ice until usage.

MuTu DC1940 cells were lysed in RIPA buffer supplemented with phosphatase and proteinase inhibitor (1:100) for 30 min on ice. Next, cells were sonicated for 15 min to shear DNA and the supernatant was cleared of cell debris by centrifugation for 10 min at 13,000 g and 4 °C. 10 µl of lysate was put aside and used as input later on. Coated beads were incubated with 500 µg cell lysate and rotated for 2 h at RT. Afterwards, the beads were washed six times in wash buffer (25 mM Tris-HCl pH 7.4, 150 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.25 % IGEPAL, 1 mM PMSF, 1 x protease inhibitor). Between each washing step, beads were rotated for 2 min at RT. After the last washing step, beads were resuspended in

SDS buffer and proteins were eluted by heating for 5 min at 95 °C. Eluted proteins were detected by western blotting.

Table 15 | Biotinylated oligonucleotides used in pulldown assay

Oligonucleotide	Sequence
5'Biotin-PD-L1 G4	5'-Biotin- TCCGCGGAGTGGGCGGGGCTCTGAACTCGAGATAAG ACCAGGAAATCGTGGTCCCCAAGCCTCATGCCAGGC TGCACTTGACGTCGCGGGCCAGTCTCCTCGCCTGC AGGTAAGGGAG
5'Biotin-scrambled	5'-Biotin- TCCGCGTAGTGCGCGACGCTCTGAACTCGAGATCAG ACGAGCAAGTCGTGATCGCGAAGCGTCATGCAACGC TGCACGTGCACGTCGCGAGCACGTCTGCTCGCTCGC GAGTGAGCGAG

2.2.26 Electromobility shift assay

Oligonucleotides (Table 16) were labelled at the 3'end with Digoxigenin (DIG) using the DIG Oligonucleotide 3'-End Labeling Kit (2nd generation, Roche). Briefly, 100 pmol oligonucleotides were mixed with the labelling reagents as indicated (Table 17) and incubated for 15 min at 37 °C. The reaction was stopped by adding 2 µl 0.2 M EDTA (pH 8.0) and stored on ice until usage.

Cell lysate was prepared as previously described (Luo et al., 2014). 5 x 10⁵ cells were pelleted and resuspended in 50 µl (5 x pellet volume) hypo-osmotic lysis buffer (0.3 M sucrose, 2 % (v/v) Tween 40, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A, 2 ng/mL leupeptin). Cells were homogenised by pipetting ~ 100 times. Samples were overlaid on 1.5 M sucrose buffer (1.5 M sucrose, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A, and 2 ng/mL leupeptin) and centrifuged for 10 min at 12,000 g and 4 °C. Supernatants were removed and the nuclear pellets were washed in low-salt wash buffer (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A,

and 2 ng/mL leupeptin). Afterwards, nuclear pellets were resuspended in 50 μ l high-salt extraction buffer (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 ng/mL pepstatin A, and 2 ng/mL leupeptin) and incubated on ice for 20 min. Finally, the samples were centrifuged for 20 min at 12,000 g and 4 °C and the supernatants were collected for further use.

A 5.5 % native polyacrylamide gel was prepared and polymerised for 1 h. The gel was prerun on ice for at least 30 min at 120 V in cold 0.25 x TBE. 1.2 μ l DIG-labeled DNA oligonucleotides were diluted in 18 μ l binding buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 150 mM KCl, 5 % Glycerol) and folded as previously described. Afterwards, an appropriate unlabelled competitor oligonucleotide (~100 x excess) was added and the samples were equilibrated to 20 °C for 5 min. Next, 2 μ l cell lysate was added and the mixture was incubated for 30 min at 20 °C before loading onto the gel. A bromphenol blue/xylene cyanol marker was used as a reference. The gel was run at 180 V for 40 min on ice, stained with 5 μ M ThT in 0.25 x TBE for 15 min and imaged to detect G4-formation using the Chemidoc MP Imaging system (BioRad). In parallel, a Hybond nylon (N+) membrane (Cytiva) was soaked in cold 0.5 x TBE for 10 min. The complexes were blotted onto the membrane at 400 mA for 40 min at RT. After rinsing the membrane with 0.5 x TBE, the membrane was dried using Whatman paper and the complexes were crosslinked to the nylon scaffold by 120 mJ/cm² using the UV Stratalinker (Stratagene). The membrane was washed for 5 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5, 0.3 % (v/v) Tween 20), followed by incubation for 30 min in blocking solution (10 x blocking solution diluted 1:10 in maleic acid buffer). Next, the membrane was incubated for 30 min in anti-DIG antibody solution (Anti-DIG AP diluted 1:10,000 in blocking solution), followed by two washing steps of 15 min in washing buffer. Finally, the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5) and incubated with CSPD working solution (CSPD diluted 1:100 in detection buffer) for 5 min prior to detection using the Chemidoc MP Imaging system (BioRad).

Table 16 | DIG-labeled oligonucleotides and competitors used for EMSA

Oligonucleotide	Sequence
PD-L1 G4_5'	GCGGAGTGGGCGGGGCTCTGAACTCGAGATAAGACCAGG AAATCGTGGTC
mSTAT1	AGCTTTCCTTTTTTCAGCT
mSTAT3	AGCTTTCAGGAAGAGCT
mSTAT1/3/5	AGCTTCATTTCCCGTAAATCCCTA

Table 17 | DIG-labeling reaction of oligonucleotides

Reagent	Volume per reaction
5x Reaction Buffer	4 µl
CoCl ₂ Solution	4 µl
DIG-ddUTP Solution	1 µl
400 U Terminal Transferase	1 µl
Oligonucleotide (100 pmol)	1 µl
Nuclease free water	9 µl
Total volume	20 µl

2.2.27 Statistical analysis

Statistical analysis was performed in GraphPad Prism 10 using parametric tests. For experiments with two groups, p-values were calculated using two-tailed student's t-tests. Analysis of two conditions in at least two independent groups was performed using multiple student's t-tests corrected for multiple comparisons with the Holm-Šidák method. Experiments with more than two groups were analysed using one-way ANOVA with Dunnett's multiple comparisons test. For experiments with multiple independent variables, a two-way ANOVA with Dunnett's or Sidak's multiple comparisons test or Fisher's least significant difference test was performed. Survival experiments were analyzed with a Log-rank/Mantel-Cox test.

3. Results - The impact of G-quadruplex stabilisation on dendritic cell phenotype and function

3.1 Introduction

DNA G4s are nucleic acid secondary structures that form in guanine-rich regions within the genome. DNA G4 structures tend to cluster in regulatory regions including telomeres, TSS and replication origin, underscoring their important homeostatic functions in telomere maintenance, transcription and DNA replication (Bochman et al., 2012; Rhodes and Lipps, 2015). G4 formation is cell type-specific and dynamically regulated by G4-interacting proteins, cell stages and exogenous stimuli. Three types of natural G4-interacting proteins have been described, including G4-binding proteins (e.g. TFs, epigenetic factors), G4-stabilising proteins (e.g. nucleolin, Zuo1) and G4 unwinding proteins such as helicases (e.g. WRN, BLM, Pif1) (Schult et al., 2023; Simon et al., 2023). So far, it is not completely understood when and how G4s form in living cells and which relevance they may have for specific cellular functions. G4 structure formation and their potential role in regulating cellular pathways has already been investigated in various cell types including stem cells and cancer cells, but little is known about G4s in immune cells such as dendritic cells. DCs are antigen-presenting cells that link innate and adaptive immunity by inducing an antigen-specific immune response. They recognise pathogenic antigens and aberrant self-antigens and process them into peptides to present those to naïve CD4⁺ and CD8⁺ T cells within the lymph nodes, resulting in T cell activation and the induction of adaptive immunity (Steinman, 2006).

In this chapter, I aimed to investigate and characterise the molecular effect of G4 formation on dendritic cells, to better understand the role of G4 structures in cellular pathways relevant for dendritic cell regulation. I assessed the molecular effect of G4 formation on DC maturation and functionality using mouse bone marrow-derived dendritic cells (BMDCs). BMDCs are primary cells that, in contrast to murine splenic DCs, can be cultured *in vitro* and thereby allow the monitoring of morphological, phenotypical and functional changes at different

stages of growth. BMDCs generated from murine stem cells in the presence of the cytokine FLT3L give rise to pDC and cDC populations. pDCs produce high amounts of Type I IFN and are therefore critical for anti-viral immunity (Cella et al., 1999; Siegal et al., 1999). cDCs comprise two subsets, cDC1 and cDC2 that can be distinguished based on a specific expression profile of cell surface markers. cDC1s are identified by their surface expression of XCR1, CD8 α and CLEC9A and can cross-present antigens and induce cytotoxic CD8⁺ T cell responses towards cancer cells, damaged cells and intracellular pathogens. In contrast, cDC2s are mainly defined by CD11b and CD172 (SIRP1 α) expression and preferentially activate several CD4⁺ helper T cell responses (Shortman and Heath, 2010).

To study G4 formation and its impact on cellular functions, several synthetic G4 ligands have been developed within the past years, which specifically bind to and stabilise G4 structures (Li et al., 2013). Some well-known DNA G4 ligands include Braco-19, RHPS4, PDS, PhenDC3, telomestatin, TMPyP4 and CX-5461. This study was conducted using the G4 stabilising ligand PDS, which targets G4 structures throughout the whole genome (Marsico et al., 2019).

DCs recognise danger signals (PAMPs and DAMPs) via PRRs on their surface, leading to antigen uptake and processing into peptides, which initiates the DC maturation process. This is associated with phenotypical and functional changes including loss of antigen-capture activity, increased expression of MHC molecules, upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 and increased production of immunomodulatory cytokines. During the maturation process, DCs also upregulate the chemokine receptor CCR7, which allows the cells to migrate to the lymph nodes, where they present antigenic peptides to naïve T cells, inducing T cell priming (Banchereau et al., 2000). In this study, I evaluated the impact of G4 formation on DC activation and maturation by assessing phenotypical and functional changes in DCs, including changes in the surface expression of the co-stimulatory molecules CD40, CD80, CD86 and MHC I and II, changes in the antigen-uptake ability, migratory capability, CCR7 regulation and cytokine production. In order to investigate the relevance of G4

formation for DC function in T cell priming, I performed co-culture experiments. T cell activation was determined based on changes in T cell activation marker expression such as CD62L, CD44, CD69 and CD25, T cell-specific cytokine production and proliferation state. Besides phenotypical and functional characterisation, I assessed the impact of G4 formation on the DC transcriptome by 3' mRNA next-generation sequencing (NGS) to identify their potential role in cellular pathways.

DCs play a crucial role in the induction of adaptive immunity, particularly in tumour immunity. Current immunotherapeutic strategies focus on boosting immune responses against cancer cells via blocking immune checkpoint molecules on the surface of immune cells with monoclonal antibodies, adoptive cell transfer, DC-based vaccines and the use of immune-stimulatory cytokines. This study will complement these data by highlighting the underlying changes in DCs induced by G4s. Moreover, it will elucidate their relevance as a potential drug target to specifically regulate DC functions on the genomic level to induce an effective anti-tumour immune response.

3.2 Results

3.2.1 Pyridostatin stabilises G-quadruplexes in bone marrow-derived dendritic cells

To characterise the molecular effect of G-quadruplex structures on DCs, murine BMDCs were used. BMDCs are stem cell-derived primary cells that can be cultured *in vitro* and allow the monitoring of morphological, phenotypical and functional changes at different stages of growth. Stem cells were isolated from the femur and tibia of C57BL/6N mice and differentiated for seven days into immature BMDCs using 200 ng/mL FLT3L. On day 7 of differentiation, immature BMDCs were treated with the G4 ligand PDS for 24 h for G4 structure stabilisation (Figure 3.1 A). PDS has been reported as a cytotoxic substance (Bossaert et al., 2021; Hou et al., 2022). As G4 stabilisation by PDS in DCs has not been studied before, I first determined the cytotoxic effect of PDS on BMDCs by performing an AlamarBlue assay. AlamarBlue measures the metabolic activity of cells, which is used to quantitatively assess cell viability and cytotoxicity.

On day 7 of differentiation, 2×10^4 cells were treated for 24 h with PDS concentrations in the range of 0 to 50 μM . Following PDS treatment, cells were incubated with 10 % AlamarBlue reagent for 4 h to determine the metabolic activity. Here, I aimed for a survival rate of ≥ 80 % to avoid possible effects based on apoptotic/necrotic mechanisms. The readout revealed a maximum concentration of 15 μM PDS (85 % viable cells), which can be used on BMDCs without major cytotoxicity (Figure 3.1 B). Immunofluorescence (IF) microscopy using the G4-specific antibody BG4 (Maurizio et al., 2024; Biffi et al., 2013) revealed that a concentration of 10 μM PDS is sufficient for robust G4 stabilisation in BMDCs by detecting nuclear G4 levels (Figure 3.1 C). BG4 signal, determined by its overall fluorescence intensity in the nuclei, was significantly increased about 1.2-fold in PDS-treated BMDCs compared to H₂O-treated BMDCs (vehicle) (Figure 3.1 D), confirming that 10 μM PDS stabilises G4 structures in DCs. Based on these data, I used 10 μM PDS for all subsequent experiments.

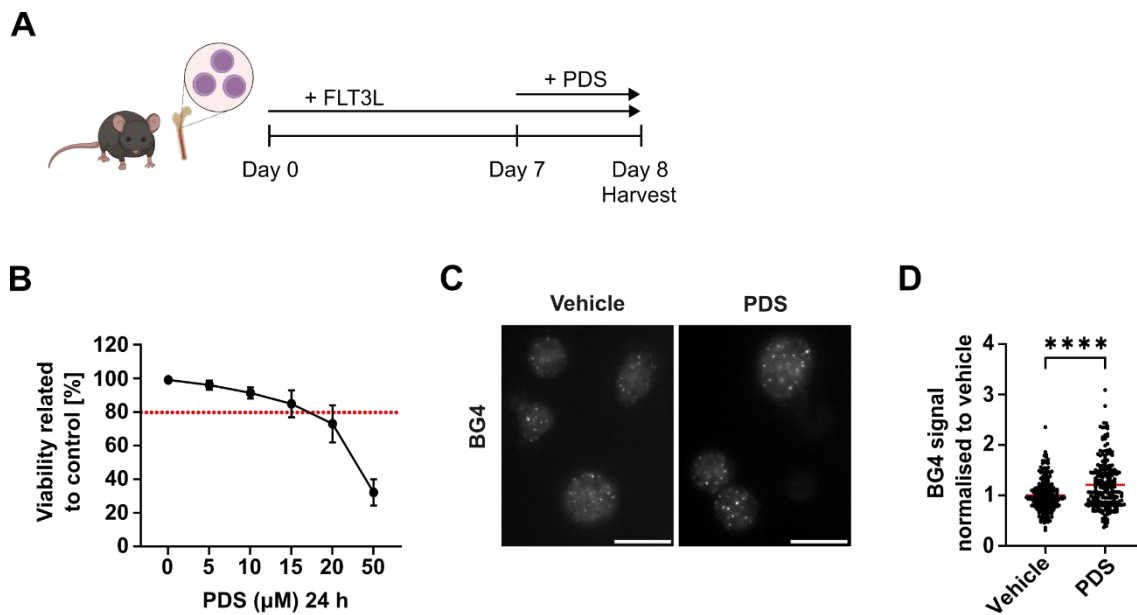


Figure 3.1 | The effect of PDS on G-Quadruplex stabilisation in BMDCs. A) Experimental setup of G-quadruplex (G4) stabilisation in bone marrow-derived dendritic cell (BMDC). Stem cells were isolated from mouse femur and tibia and differentiated into BMDCs using 200 ng/ul FMS-like tyrosine kinase 3 ligand (FLT3L). On day 7 of differentiation, immature BMDCs were treated with vehicle (H₂O) or the G4 ligand pyridostatin (PDS) for 24 h. B) PDS cytotoxicity on BMDCs was determined for 24 h using AlamarBlue. Cell viability was normalised to untreated cells (control). The red-dotted line indicates a viability threshold of 80 % in comparison to the control. Results are represented as mean $\pm$ SD based on $n = 4$ biologically independent experiments. C) Representative immunofluorescence (IF) microscopy image of G4 structures in BMDCs treated with vehicle (H₂O) or 10 μ M PDS. G4 structures were labelled with the G4-specific single-chain antibody BG4 (white signal). Magnification: 60 x, scale bar: 10 μ m. D) G4 levels determined by the fluorescent intensity of BG4 in nuclei normalised to vehicle-treated cells. The red line represents the mean value. Results are based on $n = 5$ biologically independent experiments. Significance was calculated using student's t-test. **** $p < 0.0001$.

3.2.2 PDS affects cell cycle progression, genome stability and programmed cell death in BMDCs

It has already been reported that PDS affects genome stability through the induction of DSBs e.g. via replication fork stalling, causing cell cycle arrest at the G2/M phase and the induction of apoptosis (Rodriguez et al., 2012; Zell et al., 2021; Zimmer et al., 2016). Therefore, I next investigated, if the effect of PDS on BMDCs is consistent with previous observations. I evaluated the impact of PDS

on BMDC cell cycle progression by flow cytometry using 4',6-Diamidin-2-Phenylindol (DAPI). The amount of heterochromatin present in the nuclei indicated by DAPI signal intensity allowed a distinction of the three cell cycle populations G0/1 phase, G1/S phase and S/G2 phase (Figure 3.2 A). Upon PDS treatment, the percentage of cells within the G1 phase was significantly decreased (84 % to 73 %) consistent with a comparable significant increase of cells in the G2 phase (8 % to 13 %) (Figure 3.2 B). This indicates that PDS causes BMDCs to shift from the G1 phase into G2 phase. Cells usually arrest in the G2 phase for DNA damage repair. As previously mentioned, PDS is well-known to cause DNA damage through the induction of DSBs. I assessed the impact of 10 μ M PDS on DNA damage induction in BMDCs by detecting the protein γ H2AX (the Ser139-phosphorylated form of histone H2AX), a well-known DNA damaging marker that has been reported to correlate with DSBs. Exposure to PDS led to a 1.8-fold significant upregulation in γ H2AX protein expression (Figure 3.2 C, D), showing that 10 μ M PDS causes DNA damage in BMDCs. Although the AlamarBlue assay can determine PDS cytotoxicity on BMDCs, it does not detect cells in the early apoptotic state. To further assess the effect of PDS on the induction of apoptosis, I determined the proportion of early apoptotic cells by flow cytometry using Annexin V, a phospholipid-binding protein with a high affinity for phosphatidylserine. Phosphatidylserine is a compound of the plasma membrane that, during apoptosis, is translocated from the inner to the outer surface of the plasma membrane, marking cells for recognition and phagocytosis (Demchenko, 2013). Annexin V⁺ / Live/Dead⁻ cells were classified as early apoptotic, while Annexin V⁺ / Live/Dead⁺ cells were considered late apoptotic. Live/Dead-only positive cells (Annexin V⁻ / Live/Dead⁺) were excluded from the apoptotic category and interpreted as necrotic. This classification was chosen to distinguish between apoptotic and necrotic death mechanisms, and to enable separate quantification of early and late apoptotic stages. Treatment with 10 μ M PDS resulted only in a minor increase of early apoptotic (AnnexinV⁺-only) cells (5.6 % to 8.1 %) (Figure 3.1 E, F), indicating that a concentration of 10 μ M PDS does not greatly affect the induction of apoptosis in BMDCs.

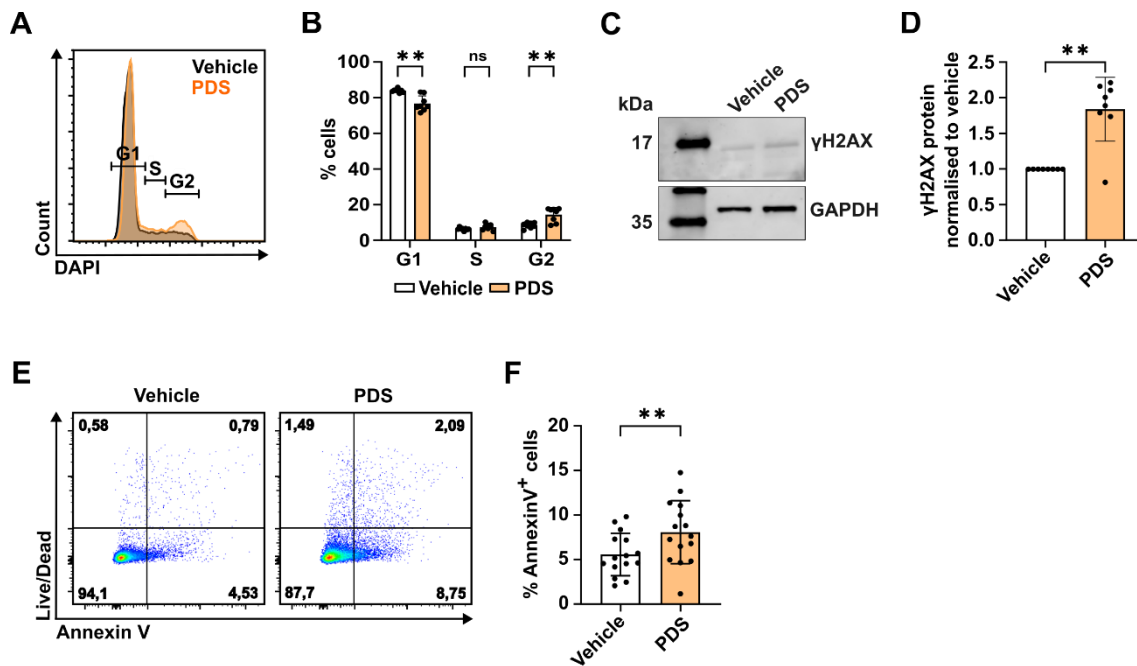


Figure 3.2 | Impact of PDS on cell cycle progression, genome stability and apoptosis in BMDCs. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H₂O) or 10 μ M pyridostatin (PDS) for 24 h. A) Representative histogram showing cell cycle distribution of vehicle (black) and PDS (orange)-treated BMDCs. The three cell cycle phases G1, S and G2 were distinguished based on the DAPI signal intensity. B) Percentage of vehicle- or PDS-treated BMDCs distributed in G1, S and G2 phase. Data are represented as mean $\pm$ SD of $n = 3$ independent experiments. C) Representative western blot of the DNA damaging marker γ H2AX (15 kDa) in vehicle- and PDS-treated BMDCs. GAPDH (38 kDa) was used as a loading control. D) Quantification of γ H2AX protein expression. Fold change was determined by normalising γ H2AX signal intensity over GAPDH (loading control) and further over vehicle-treated cells. Data are represented as mean $\pm$ SD of $n = 3$ independent experiments. E) Representative scatter plots display the proportion of apoptotic cells in vehicle- and PDS-treated BMDCs. Annexin V⁺ cells are classified as early apoptotic cells, while Annexin V⁺/Live/Dead⁺ are considered late apoptotic cells. Live/Dead⁺ are interpreted as necrotic. F) Percentage of Annexin V⁺ cells within vehicle- and PDS-treated BMDCs. Data are represented as mean $\pm$ SD of $n = 5$ independent experiments. Significance was calculated using multiple student's t-test corrected for multiple comparisons using the Holm-Šidák method (B) and single student's t-tests (D, F). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.3 G4 stabilisation by PDS induces activation of murine BMDCs and human monocyte-derived dendritic cells

DCs recognise PAMPs and DAMPs via PRRs on their surface (Diebold, 2009). The recognition of “danger signals” in response to invading pathogens or damaged self triggers the DC maturation and activation process, which is required for the induction of an appropriate immune response. DC activation is associated with phenotypical changes, including increased expression of MHC class I (MHC I) and II (MHC II) molecules as well as upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 (Banchereau et al., 2000).

In order to characterise the molecular effect of G4 formation on DC activation, I examined changes in the expression of surface markers characteristic for activated DCs (amongst CD40, CD80, CD86, MHC I and MHC II) on BMDCs following PDS treatment. Due to their major role in inducing inflammatory and anti-tumour immune responses, I focused on phenotypical changes of conventional DCs, which can be subdivided into cDC1s and cDC2s. cDC1s are determined by CD11c⁺B220⁻XCR1⁺CD11b^{intermediate} expression, while cDC2 are characterised as CD11c⁺B220⁻XCR1⁻CD11b⁺ cells (Figure 3.3 A).

To compare possible changes in surface expression levels with those of fully activated DCs (positive control), BMDCs were stimulated with 1 µM CpG ODN 1668 (Class B), a synthetic unmethylated oligodeoxynucleotide comprised of CpG motifs that are highly prevalent in bacterial DNA (Wagner, 1999). CpG ODNs are recognised by TLR9 (Hemmi et al., 2000).

With PDS, the proportion of mature DCs, indicated by CD11c⁺MHCII⁺ cells, was significantly enriched (33 % to 52 %) compared to vehicle-treated BMDCs. Moreover, the amount of mature PDS-treated BMDCs was equal to mature CpG-stimulated BMDCs (Figure 3.3 B, C).

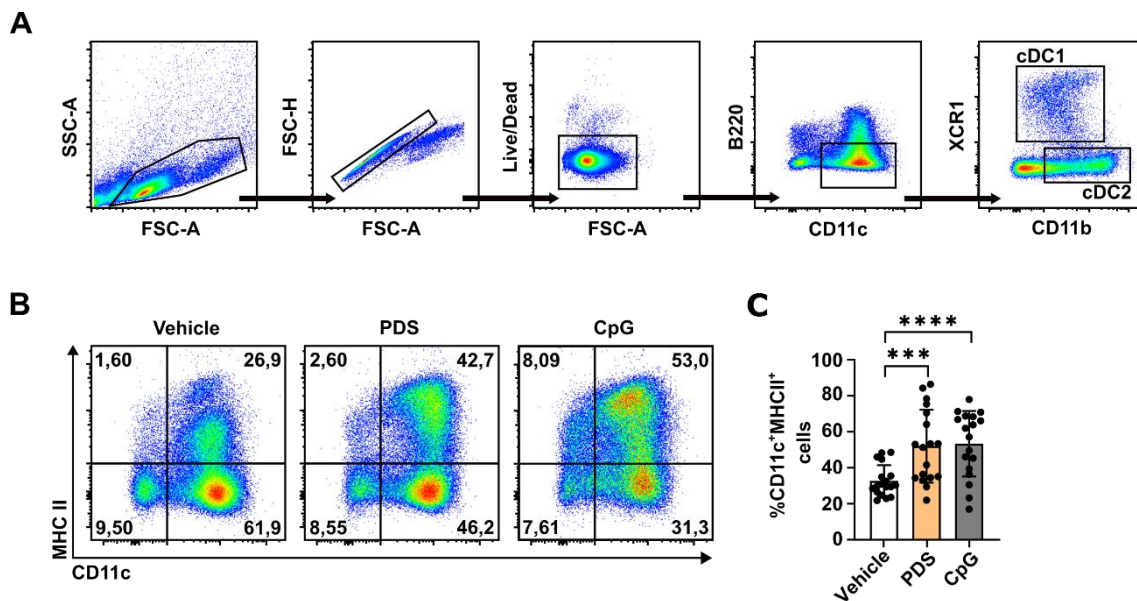


Figure 3.3 | PDS induces DC maturation. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H₂O), 10 μ M pyridostatin (PDS) or 1 μ M CpG (positive control) for 24 h. Cells were stained with mAbs specific for markers of conventional DC (cDC) subsets and DC activation. DC surface marker expression was assessed by flow cytometry. A) Representative gating strategy for BMDCs. cDCs were distinguished from pDCs through gating on CD11c⁺B220⁻ expressing cells. cDC1s are determined as CD11c⁺B220⁻XCR1⁺CD11b^{intermediate}, while cDC2 are characterised as CD11c⁺B220⁻XCR1⁻CD11b⁺. B) Representative scatter plots display the proportion of CD11c⁺MHCII⁺ cells in vehicle-, PDS- and CpG-treated BMDCs. C) Percentage of CD11c⁺MHCII⁺ cells in vehicle-, PDS- and CpG-treated BMDCs. Data are represented as mean $\pm$ SD of n = 6 independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (C). ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Concurrently, PDS resulted in elevated surface expression of MHC I and MHC II as well as CD40, CD80 and CD86 in both, cDC1s and cDC2s (Figure 3.4). However, surface expression was not as strongly upregulated as on CpG-stimulated BMDCs, especially in cDC1s (Figure 3.4 A). Surface expression of CD40, CD80 and MHC I was increased to a similar extent in cDC1s and cDC2s (CD40: 2.7- to 3-fold; CD80: 2.5-fold, MHC I: 1.7- to 2.2-fold). In contrast, with PDS, CD86 and MHC II were increased to a higher level in cDC2s (CD86: 2.9-fold (cDC1) vs 5.5-fold (cDC2); MHC II: 1.5-fold (cDC1) vs 3.8-fold (cDC2)) (Figure 3.4 B), suggesting that the molecules are differentially regulated within

the cDC subsets. Additionally, surface expression levels of CD80, MHC I and MHC II on cDC2s following PDS treatment were comparable to CpG-stimulated cDC2s (CD80: 2.6- to 2.8-fold; MHC I: 2.2- to 2.6-fold; MHC II: 3.8-fold). Together, these results demonstrate that G4 stabilisation by PDS triggers DC activation through the upregulation of DC activation marker surface expression.

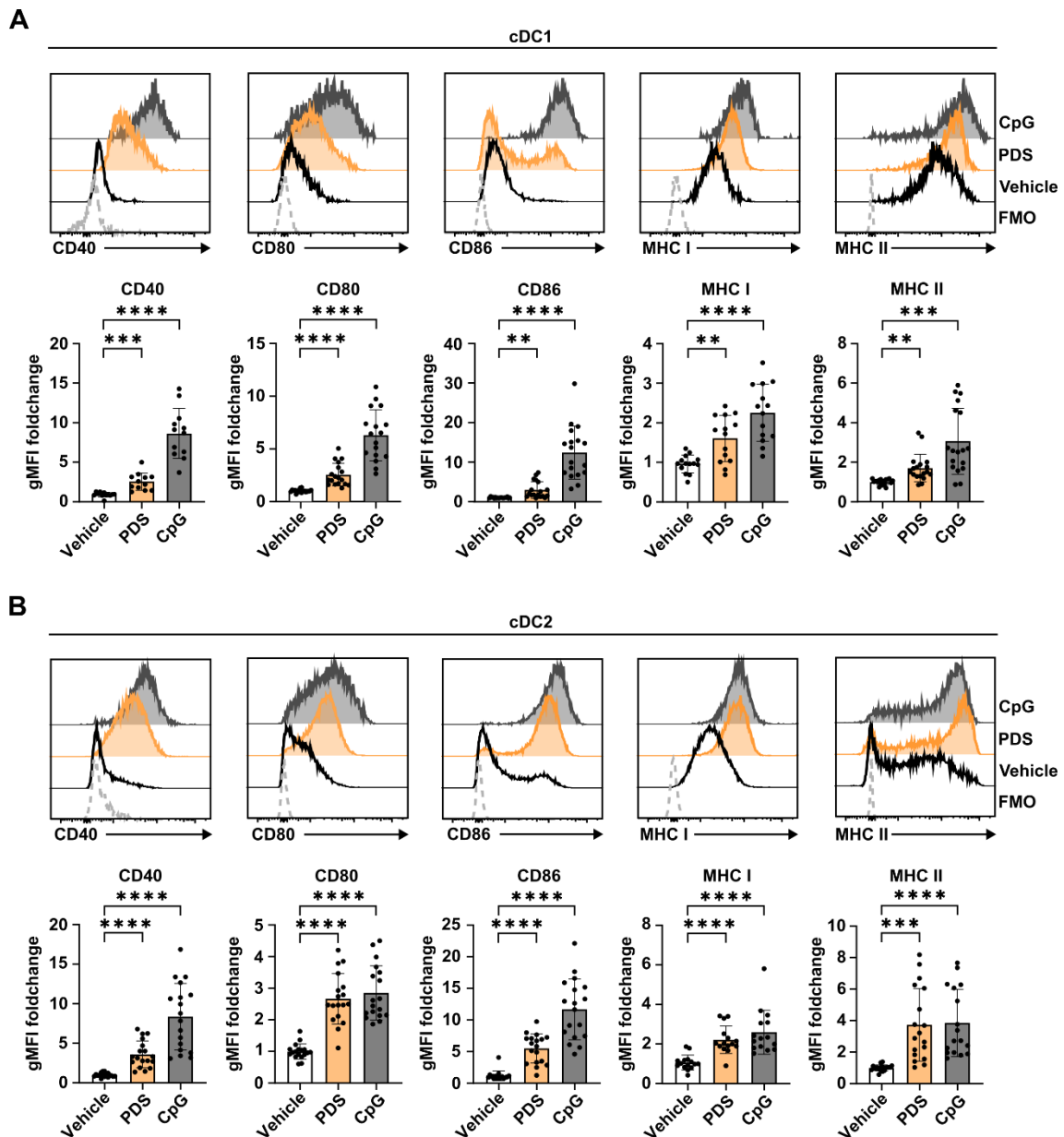


Figure 3.4 | PDS induces DC activation marker expression on BMDCs. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H₂O), 10 μ M pyridostatin (PDS) or 1 μ M CpG (positive control) for 24 h. BMDCs were stained with mAbs specific for markers of DC activation. DC surface marker expression was assessed by flow cytometry.

Representative histograms showing the distribution of the co-stimulatory molecules CD40, CD80 and CD86 and major histocompatibility complex (MHC) I and II molecules on cDC1s (A) and cDC2s (B) following treatment with vehicle (H₂O), PDS or CpG. Graphs display the differences in surface expression, depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean $\pm$ SD of n = 6 independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (A, B). ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

In order to transfer previous findings with murine BMDCs to the human system, I assessed the impact of G4 stabilisation on human monocyte-derived DCs (MoDCs). Monocytes isolated from buffy coats were differentiated into MoDCs for seven days using the cytokines GM-CSF and IL-4. On day 6 of differentiation, cells were treated with PDS for 24 h (Figure 3.5 A). Viability testing revealed a maximum concentration of 25 μ M PDS (80.5 % viable cells) that can be used on MoDCs to obtain a survival rate > 80 % (Figure 3.5 B). To match the conditions with those of 10 μ M PDS in BMDCs, MoDCs were also treated with 10 μ M PDS (88 % viable cells) for 24 h. It has been reported by Hartmann et al. (1999) that MoDCs are not activated by CpG ODN. Therefore, cells were stimulated with 100 ng/mL LPS as a positive control for activated MoDCs. LPS is a major component of the outer cell membrane of Gram-negative bacteria, such as *E. coli* and *Salmonella* and is recognised by TLR4 (Avila-Calderón et al., 2021).

MoDCs are characterised as CD14⁻CD11c⁺MHCII⁺ cells. Here, I evaluated changes in the surface expression of CD40, CD80 and CD86 following PDS treatment. Similar to BMDCs, PDS resulted in a significant upregulation of CD40 (1.25-fold) and CD80 (1.5-fold) on MoDCs (Figure 3.5 C). Expression of CD86 was not affected by PDS. LPS stimulation led to a 2- to 3-fold enhanced surface expression compared to PDS treatment. Together, these data show that PDS induces the expression of DC activation markers in murine and human systems. However, it should be taken into account that the effects, especially in the human system, were only modest (in terms of magnitude).

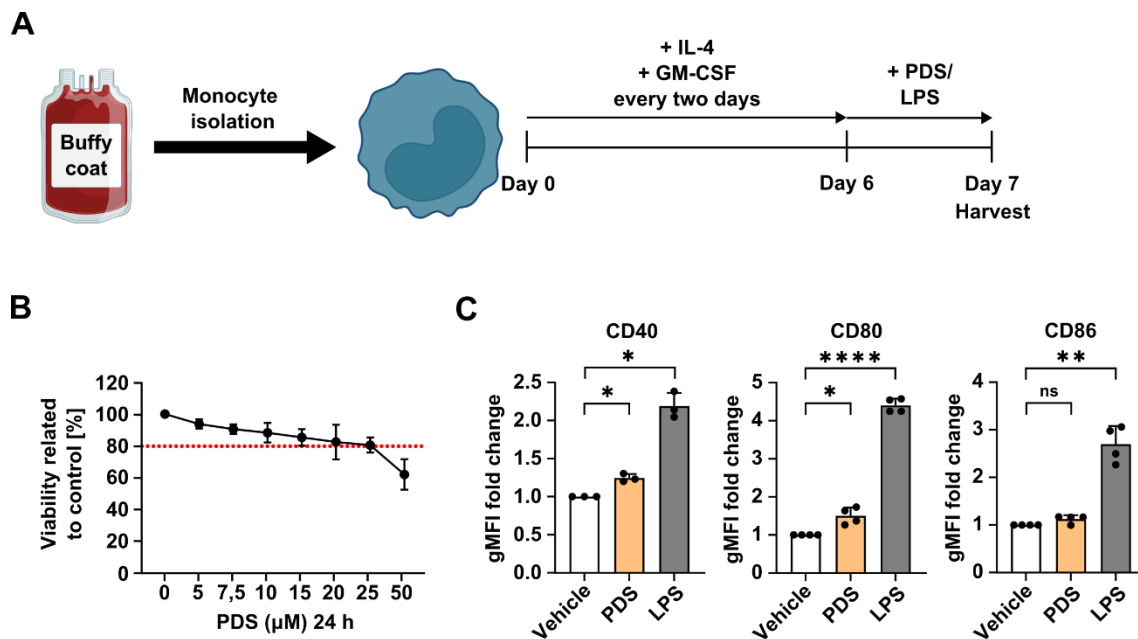


Figure 3.5 | PDS stimulates the surface expression of DC activation markers on human MoDCs. A) Experimental setup of human monocyte-derived DC (MoDC) differentiation. Monocytes were isolated from buffy coats and differentiated into MoDCs using IL-4 and GM-CSF. On day 6 of differentiation, MoDCs were treated with vehicle (H_2O), pyridostatin (PDS) or lipopolysaccharide (LPS; positive control) for 24 h. B) PDS cytotoxicity on MoDCs was determined for 24 h using AlamarBlue. Cell viability was normalised to untreated cells (control). The red-dotted line indicates a viability threshold of 80 % in comparison to the control. Results are represented as mean $\pm$ SD based on $n = 4$ biologically independent experiments. C) Surface expression of the co-stimulatory molecules CD40, CD80 and CD86 on MoDCs following treatment with 10 μM PDS or 100 ng/mL LPS for 24 h. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean $\pm$ SD based on $n = 4$ biologically independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.4 PDS induces dendritic cell migration, but does not affect antigen uptake and cytokine secretion

Besides phenotypical changes, including the upregulation of co-stimulatory and MHC molecules, DC maturation is associated with functional changes including loss of antigen-capture activity, increased production of immunomodulatory cytokines and upregulation of CCR7, which allows the cells to migrate to lymph

nodes, where they present antigenic peptides to naïve T cells (Banchereau et al., 2000).

In order to characterise the effect of G4 formation on DC maturation, I examined changes in the surface expression of CCR7, migration capacity, cytokine secretion and antigen-capture ability in BMDCs following PDS treatment.

Mature DCs upregulate the chemokine receptor CCR7, which allows the cells to migrate to the lymph nodes via CCL19 and CCL21 (Förster et al., 1999; Gunn et al., 1999; Jang et al., 2006). Upon PDS treatment, CCR7 expression was significantly upregulated about 5-fold in comparison to vehicle-treated BMDCs (Figure 3.6 A, B). CpG stimulation revealed a 14-fold increase in CCR7 expression. CCR7 expression usually correlates with DC migration. To determine, if the increased expression of CCR7 affects DC migration, I performed a transwell migration assay. 2×10^5 cells were allowed to migrate towards a CCL19 chemokine gradient for 3 h. The number of migrated cells was calculated by flow cytometry using counting beads. Following PDS treatment, the number of cells migrated towards CCL19 was significantly enriched (5.9×10^4) compared to the vehicle-treated control (4.7×10^4) (Figure 3.6 C). With CpG stimulation, even more cells (9.8×10^4) migrated towards the chemokine gradient. Together with the upregulation of CCR7, these data reveal that PDS enhances DC migration.

Next, I assessed the secretion of inflammatory cytokines (IL-12p70, IL-6, TNF- α , IL-17, IL-1 α , IL-1 β , IL-23, IL-10, MCP-1 and IFN- β) by DCs 24 h following PDS stimulation in the cell supernatant with the cytometry-based multiplex immunoassay LEGENDplex™ (BioLegend). However, no differences in cytokine secretion levels between vehicle- and PDS-treated BMDCs were detected (data not shown).

Another readout for DC maturation is the loss in antigen-capture activity, which I determined by the use of FITC-labelled dextran. Following treatment with PDS for 24 h, 2×10^5 BMDCs were incubated with 1 mg/mL FITC-Dextran for up to 90 min. I assessed the FITC-Dextran uptake by flow cytometry. PDS-treated BMDCs revealed 15 % less dextran uptake in comparison to vehicle-treated BMDCs after

90 min of incubation, showing that PDS only slightly affects the ability of antigen uptake (Figure 3.6 D)

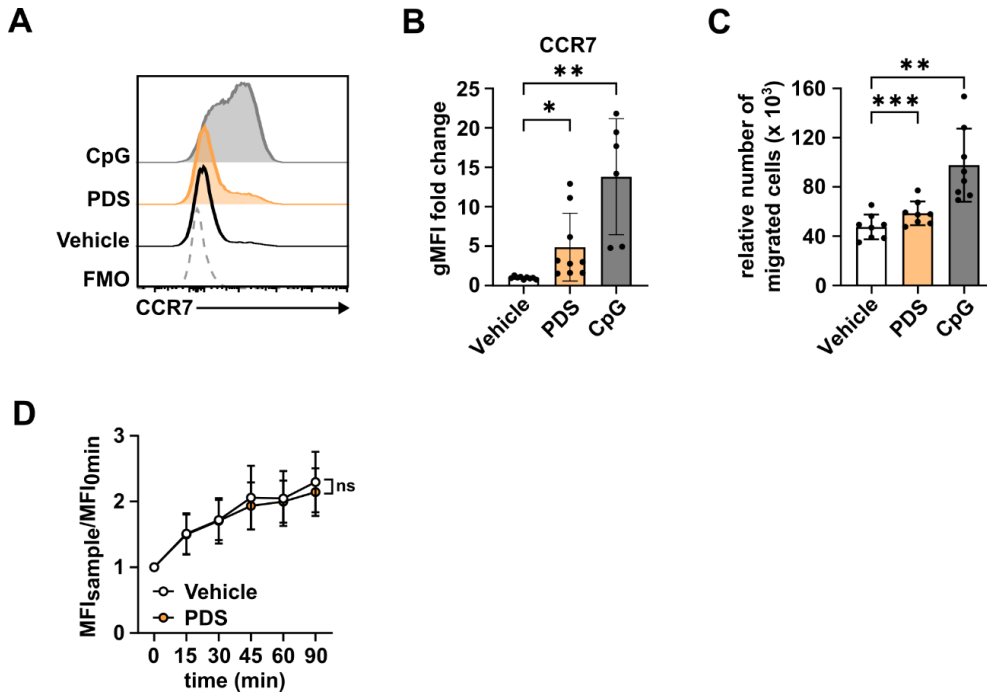


Figure 3.6 | The effect of PDS on DC migration and antigen uptake. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H_2O) 10 μM pyridostatin (PDS) or 1 μM CpG (positive control) for 24 h. A) Representative histogram showing the proportion of CCR7⁺ cells in vehicle-, PDS- and CpG-stimulated BMDCs. FMO: fluorescent minus one control. B) CCR7 surface expression is depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean $\pm$ SD based on $n = 3$ independent experiments. C) Number of vehicle-, PDS- and CpG-treated BMDCs migrated towards a CCL19 gradient for 3 h using transwell chambers. Data are represented as mean $\pm$ SD based on $n = 4$ independent experiments. D) FITC-Dextran uptake by vehicle- and PDS-treated BMDCs. BMDCs were incubated with FITC-Dextran for 15, 30, 45, 60 and 90 min at 37 $^\circ\text{C}$ and 90 min on ice (control). Dextran uptake is shown as MFI_{sample} normalised to MFI_{0min}. Data are represented as mean $\pm$ SD based on $n = 3$ independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (B, C) and two-way ANOVA with Sidak's multiple comparisons test (D). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.5 G4 stabilisation by Braco-19 induces DC activation similar to PDS

In the past years, several synthetic G4 ligands have been developed, which specifically bind to and stabilise G4 structures to facilitate the investigation of G4 formation and their impact on cellular functions (Li et al., 2013). Some well-known DNA G4 ligands include Braco-19, TMPyP4 and CX5461. Here, I investigated, if these compounds affect DC activation similar to PDS.

Braco-19 is one of the G4 ligands with specific telomerase inhibitory activity by targeting G4s in telomeres (Burger et al., 2005). TMPyP4 is considered to be another potent telomerase inhibitor as it binds to and stabilises G4s in human telomeric DNA, thus resulting in telomerase inhibition (Han et al., 1999; Shi et al., 2001; Wheelhouse et al., 1998). Moreover, TMPyP4 has been reported to downregulate the expression of oncogenes, such as c-MYC and KRAS through the stabilisation of G4 structures in the respective gene promoter regions (Cogoi and Xodo, 2006; Siddiqui-Jain et al., 2002). CX-5461, originally identified as an RNA polymerase I inhibitor, is also used as a G-quadruplex stabiliser, causing DNA damage and G2-phase arrest by blocking replication forks and inducing DNA breaks (Xu et al., 2017).

First, I determined the cytotoxic effect of Braco-19, CX5461 and TMPyP4 on BMDCs for 24 h using AlamarBlue. The viability assay revealed that concentrations up to 60 μ M Braco-19, 100 nM CX-5461 and 60 μ M TMPyP4 could be tolerated to keep the survival rate of BMDCs above the viability threshold of 80 % (Figure 3.7 A). To match the conditions with those of 10 μ M PDS, which resulted in 90 % cell survival (Figure 3.1 B), BMDCs were treated with 20 μ M Braco-19, 40 nM CX-5461 and 20 μ M TMPyP4.

In order to confirm G4 stabilisation by these G4 ligands, I determined G4 levels by IF microscopy using the BG4 antibody (Figure 3.7 B). BG4 signal intensity was significantly increased in BMDCs treated with Braco-19 (1.3-fold) and CX-5461 (1.24-fold) compared to vehicle (Figure 3.7 B, C), confirming that these ligands stabilise G4 structures in BMDCs. With TMPyP4, no difference in BG4 signal intensity was observed.

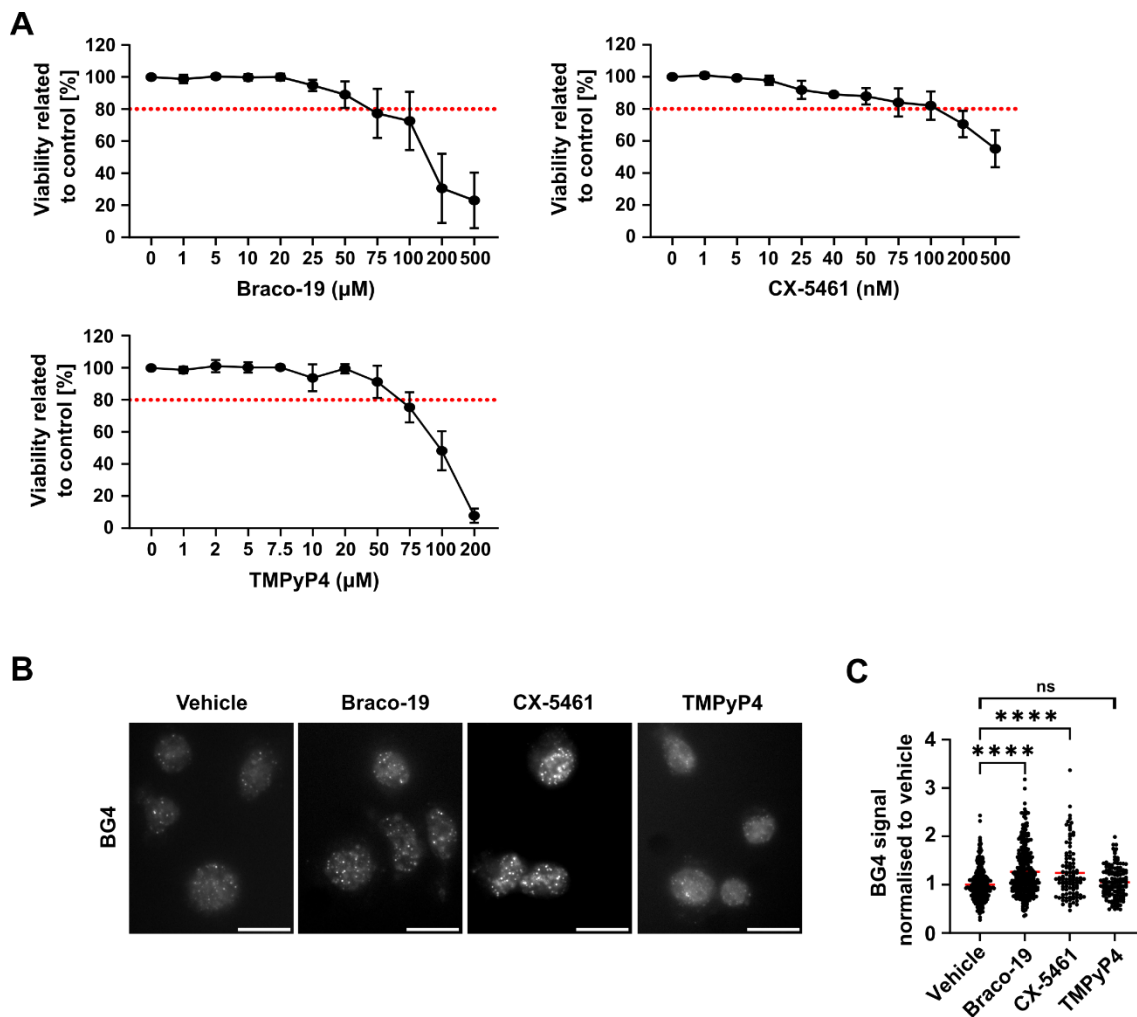


Figure 3.7 | The effect of Braco-19, CX-5461 and TMPyP4 on G4 stabilisation in BMDCs. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with the G-quadruplex (G4) ligands Braco-19, CX-5461 and TMPyP4 for 24 h. A) Cytotoxicity of Braco-19, CX-5461 and TMPyP4 on BMDCs was determined using AlamarBlue. Cell viability was normalised to untreated cells (control). The red-dotted line indicates a viability threshold of 80 % in comparison to the control. Results are represented as mean $\pm$ SD based on $n = 4$ biologically independent experiments. B) Representative immunofluorescence (IF) microscopy image of G4 structures in BMDCs treated with 20 μM Braco-19, 40 nM CX-5461, 20 μM TMPyP4 or vehicle (H_2O) for 24 h. G4 structures were labelled with the G4-specific single-chain antibody BG4 (white signal). Magnification: 60 x, scale bar: 10 μm . C) G4 levels determined by the fluorescent intensity of BG4 in nuclei normalised to vehicle-treated cells. The red line represents the mean value. Results are based on $n = 2-4$ biologically independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To characterise the molecular effect of Braco-19, CX-5461 and TMPyP4 on DC activation, I next examined changes in the surface expression of the DC markers CD40, CD80, CD86 and MHC II on cDC1 and cDC2 BMDCs following treatment with the G4 ligands. Braco-19 led to a significant upregulation of CD40, CD80 and CD86 on both cDC1s (CD40: 3.2-fold, CD80: 1.4-fold, CD86: 2.8-fold) and cDC2s (CD40: 2.3-fold, CD80: 1.5-fold, CD86: 2.9-fold) (Figure 3.8). With Braco-19, expression levels of CD40 and CD80 were comparable to those of PDS-treated BMDCs, while CD86 was even more elevated with Braco-19. Comparable to PDS, Braco-19 treatment resulted in a 1.5-fold upregulation of MHC II on cDC2s. CX-5461 only caused a significant upregulation of CD80 in cDC1s (1.4-fold) and cDC2s (1.9-fold) (Figure 3.8). In contrast, TMPyP4 induced expression of CD40 and MHC II in cDC1s (CD40: 5.8-fold, MHC II: 3.2-fold) and cDC2s (CD40: 3.6-fold, MHC II: 4.3-fold) to a significantly higher level than PDS (Figure 3.8). In addition, also expression of CD86 was elevated (cDC1: 1.5-fold, cDC2: 1.8-fold) in TMPyP4-treated BMDCs, only CD80 was not affected by the ligand. Together, these data indicate that other G4 ligands, besides PDS, influence the regulation of DC activation markers.

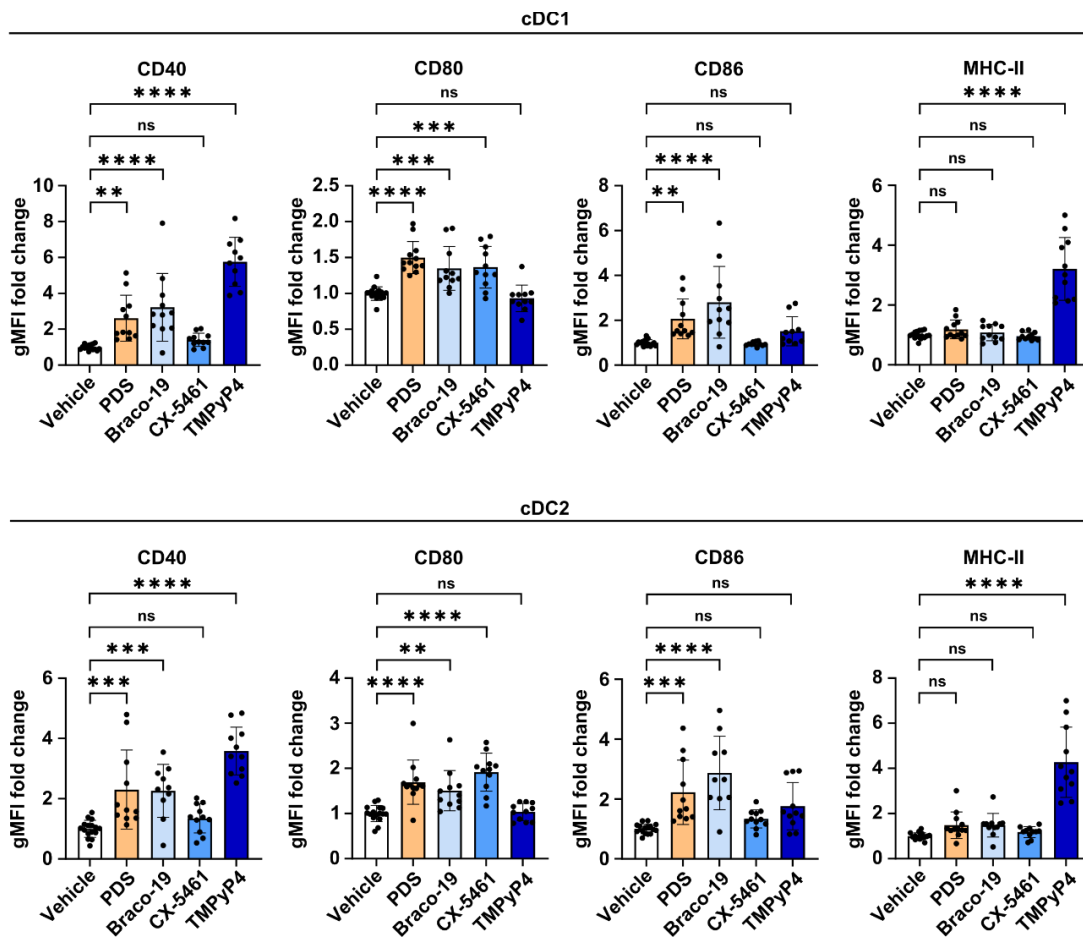


Figure 3.8 | Braco-19, CX-5461 and TMPyP4 affect the surface expression of DC activation markers. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H₂O), 10 μ M pyridostatin (PDS), 20 μ M Braco-19, 40 nM CX-5461 and 20 μ M TMPyP4 for 24 h. Cells were stained with mAbs specific for markers of conventional dendritic cell (cDC) subsets and DC activation. DC surface marker expression was assessed by flow cytometry. Graphs display changes in the surface expression of the co-stimulatory molecules CD40, CD80 and CD86 and major histocompatibility complex (MHC) II on cDC1s and cDC2s following treatment with PDS, Braco-19, CX-5461 and TMPyP4 for 24 h. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean $\pm$ SD based on n = 4 independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3.2.6 G4 stabilisation induces transcriptional reprogramming in BMDCs

Besides phenotypical and functional characterisation, I further wanted to investigate if G4 stabilisation by PDS leads to transcriptomic reprogramming in DCs and if the previous observations can be attributed to such transcriptomic changes.

Stabilisation of DNA G-quadruplexes can have various consequences on the transcriptional level, depending on their location and which processes are affected by G4 formation. Rodriguez et al. (2012) reported that treatment with PDS causes DNA damage at G4 sites in a transcription- and replication-dependent manner, resulting in gene downregulation. This phenotype was attributed to replication fork stalling due to G4 formation leading to single- or double-strand breaks and genome instability. DNA damage and genome instability, in turn, are also connected with the upregulation of genes involved in DNA repair and apoptosis (Karakaidos et al., 2020). Moreover, G4 structures are highly overrepresented in gene promoter regions (Huppert and Balasubramanian, 2007; Rhodes and Lipps, 2015). Depending on which strand G4s are forming within the transcription bubble, they can differentially affect gene transcription. G4 structures on the template strand rather act as transcriptional repressors by impeding TF binding to duplex-DNA or RNA polymerase stalling. In contrast, G4 formation on the coding strand was reported to enhance the transcription of particular genes by favouring the binding of TFs or by maintaining the DNA molecule in a single-stranded confirmation, thus facilitating the re-initiation of transcription (Bochman et al., 2012).

To identify potential G4-dependent transcriptomic changes, BMDCs were generated as previously described and treated either with 10 μ M PDS or H₂O (vehicle) for 24 h on day 7 of differentiation. Afterwards, pan-DCs, including pDCs and cDCs, were sorted from bulk BMDCs and total RNA was isolated. Using 3' mRNA sequencing, conducted by the NGS Core Facility (University of Bonn), I evaluated the impact of G4 stabilisation by PDS on the DC transcriptome.

Comparative RNA sequencing analysis of the two groups, vehicle- and PDS-treated BMDCs, identified 595 significant differentially expressed genes (DEGs) (adjusted p-value < 0.05). From these DEGs, 475 genes were significantly upregulated and 120 genes were significantly downregulated following PDS treatment. The gene showing the highest upregulation of expression between the two groups was *Mx1* (Figure 3.9 A). *Mx1* is an interferon-induced guanosine triphosphate (GTP)-binding protein that plays a central role as an effector in the cellular response against multiple viruses (Haller et al., 1995; Pavlovic et al., 1992; Thimme et al., 1995). Other interferon-stimulated genes (ISGs) involved in anti-viral defence response that were significantly upregulated through PDS include *Ifih1* (MDA-5), *Irf7*, *Ifit1*, *Ifit2*, *Mx2*, *Rsad2* and *Rtp4* (Figure 3.9 A), which are associated with viral sensing and inhibition of viral replication (Schoggins and Rice, 2011). pDCs are the main players in cellular anti-viral defence through rapid and massive production of type I IFN (Cella et al., 2000). Therefore, these results suggest that PDS triggers pDC activation, thus inducing an anti-viral immune response by stimulating the expression of the above-mentioned anti-viral genes. Another cluster of genes that were significantly upregulated in PDS-treated BMDCs compared to vehicle include *IL-6*, *CD83*, *IL-12b*, *IL-1b*, *CCL5* and *Signal transducer and activator of transcription 1 (STAT1)* (Figure 3.9 A). These genes are critical for DC maturation, antigen presentation, chemoattraction and T cell differentiation (Aerts-Toegaert et al., 2007; Bettelli et al., 2006; Heufler et al., 1996; Jackson et al., 2004; Rawat et al., 2023). Examples of genes that were significantly downregulated with PDS are *Hepacam2*, a protein that plays a role in mitosis, the transcription factor *KLF4*, H3 clustered histone 10 (*H3c10*) and the transcriptional corepressor *transducin-like enhancer 2 (TLE2)*. In contrast to the significantly upregulated genes, no gene cluster of significantly downregulated genes could be identified.

In addition, I subjected the significantly regulated genes to Gene ontology (GO) analysis, in order to characterise the underlying biological processes. The Top10 pathways identified include “defence response” (161 DEGs), “response to external stimulus” (205 DEGs), “innate immune response” (107 DEGs), “response to virus” (73 DEGs), “response to stress” (234 DEGs), “cellular

response to cytokine stimulus" (100 DEGs), "response to bacterium" (86 DEGs), "cytokine production" (73 DEGs), "cell activation" (76 DEGs) and "lymphocyte activation" (61 DEGs) (Figure 3.9 B). "Response to virus" revealed the strongest enrichment (7.49-fold enriched). This is in line with the various anti-viral genes that were identified within the Top100 DEGs (Figure 3.9 A), indicating that PDS strongly affects the anti-viral immune response. Importantly, all positively regulated biological processes are related to DC activation and their function to induce an appropriate immune response.

As my previously described functional data points towards the activation of DCs following PDS treatment, I investigated DEGs associated with DC activation in more detail in BMDCs. DEGs linked to DC activation include the chemokines *CXCL2/9/10/11* and *CCL3/4/5*, the cytokines *IL-12b*, *IL-6*, *IL10*, *IL1 β* , the surface markers *CCR7*, *CD40*, *CD80*, *CD83* and *CD86* as well as *STAT1* and *Suppressor of cytokine signalling 2 (Socs2)* (Figure 3.9 C). The strongest upregulation in this set was detected for *STAT1*, a central factor required for DC maturation (Jackson et al., 2004) and *Socs2*, which in DCs is responsible for positive regulation of T cell activation (Hu et al., 2012). Based on these results, it can be suggested that DC activation by PDS-dependent G4 stabilisation is triggered by transcriptional reprogramming, regulating several stimulatory pathways. Particularly, these data indicate that changes in co-stimulatory surface proteins such as *CD40*, *CD80*, *CD86* are regulated on the transcriptional level.

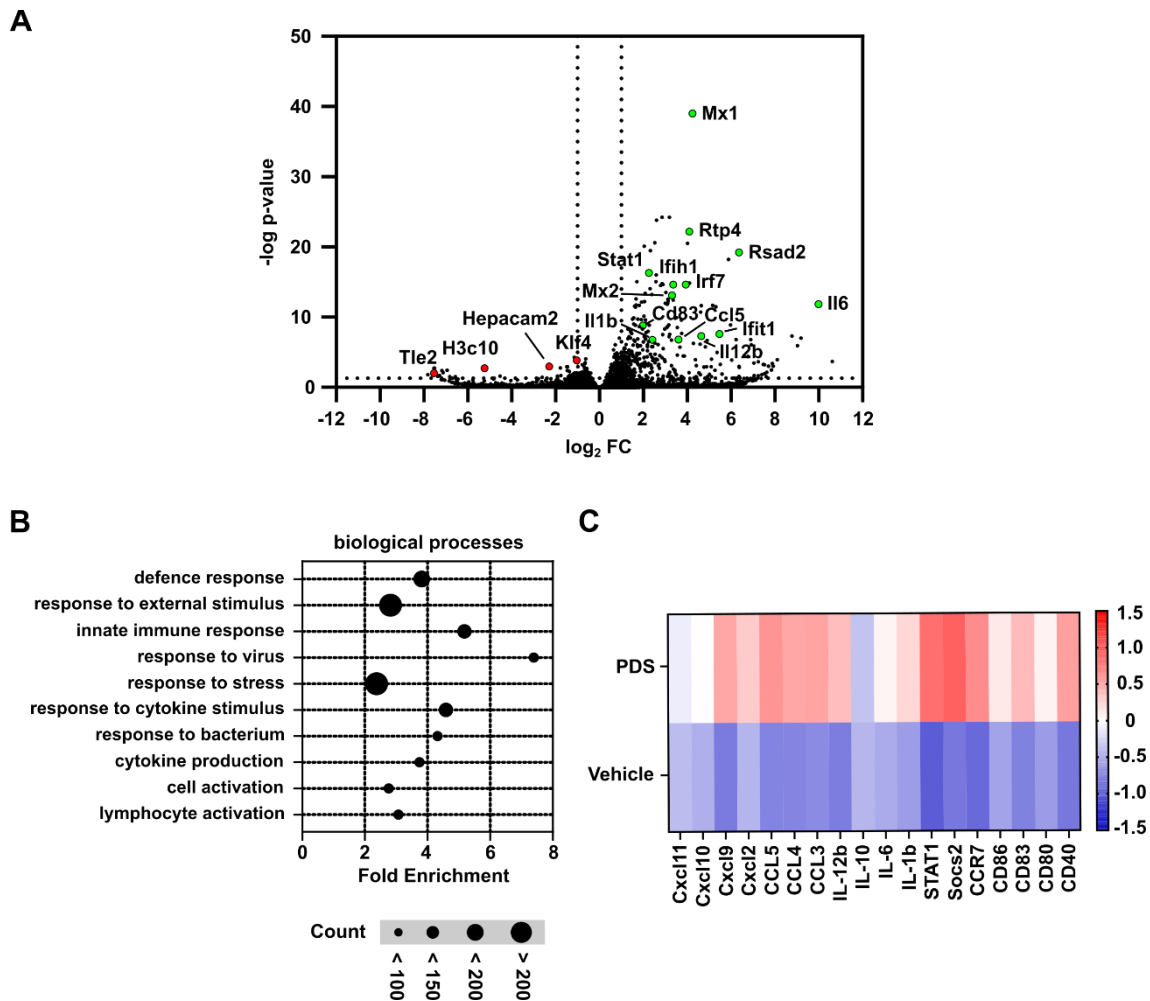


Figure 3.9 | Transcriptomic signature of PDS-treated BMDCs. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H_2O) or 10 μM pyridostatin (PDS) for 24 h. Pan-DCs were isolated from bulk BMDCs. Total RNA was isolated from these cells and subjected to 3' mRNA next-generation sequencing (NGS). A) Volcano plot showing selected genes of the Top 100 differentially expressed genes (DEGs). Upregulated genes are highlighted in green and downregulated genes in red. B) The Top 10 biological processes underlying DEGs. The size of bubbles corresponds to the relative number of DEGs overlapping with the pathway. C) Heatmap of DEGs involved in DC activation. Data display the z-scores of DEGs from vehicle- and PDS-treated BMDCs. The z-scores were calculated for each gene based on DESeq2-normalised counts.

3.2.7 The impact of PDS on BMDCs is not DNA damage-mediated

It is well-known that PDS induces DNA DSBs in cell lines (Rodriguez et al., 2012). In addition, I have previously shown that PDS induces DNA damage in BMDCs,

too (Figure 3.2 C, D). Therefore, I next investigated, if the previous observations on BMDC activation and maturation stimulated by PDS are based on PDS-mediated DNA damage. DNA DSBs are repaired by two major pathways: HR and NHEJ. A key determinant of repair pathway choice is the cell cycle phase. HR is a template-directed repair process that takes advantage of a homologous sequence to ensure accurate repair of the DNA break (Kowalczykowski, 2015). Therefore, HR is highly active during the S and G2 phases of the cell cycle, when a homologous template in the form of the sister chromatid is present. NHEJ mediates direct re-ligation of the broken DNA ends (Davis and Chen, 2013). Unlike HR, NHEJ does not require a homologous template for the repair of DNA lesions and is hence not restricted to a certain phase of the cell cycle.

3' mRNA sequencing revealed no significant changes in the expression of DNA damage repair genes involved in the HR or NHEJ pathway between vehicle- and PDS-treated BMDCs (Table 18). This indicates that 10 μ M PDS does not induce the expression of repair proteins required for DNA DSBs.

Table 18 | Genes involved in DNA repair of DSBs

Pathway	Gene	Log ₂ FC	adj p-value
HR	Mre11a	0,69886571	0,62778205
	Rad50	0,1064274	0,9390205
	Rbbp8	0,15572957	0,92247032
	Rpa1	-0,09476641	0,93824159
	Rad51	-0,13878243	0,94956575
	Brca2	0,38034357	0,84154066
	Brca1	0,27862959	0,87860877
	Xrcc3	-0,04515423	0,99628527
	Rad54b	0,50066679	0,87418826
	Rad51c	-0,05048856	0,99510393
	Blm	0,05460372	0,98938099
	Top3a	0,2576179	0,95605863
	Rmi1	-0,67217086	0,67449797
	Rmi2	0,45974852	0,88985691
	Mus81	0,16473315	0,96354957
	Eme1	0,11923683	0,95906094
	Gen1	-0,33404467	0,90977847
	Dna2	-0,37512289	0,85062865
	Exo1	0,58639723	0,8908289
NHEJ	Xrcc6	-0,87585187	0,49658941
	Prkdc	-0,16918002	0,97387908
	Dclre1c	0,43091454	0,40248923
	Xrcc4	1,10906748	0,71522717
	Lig4	-0,79337332	0,63769189
	Parp3	0,64164938	0,11537831

To assess the impact of DNA damage on PDS-mediated DC activation, BMDCs were treated with hydroxyurea (HU). Like PDS, HU causes replication fork stalling, leading to DNA DSBs (Petermann et al., 2010). Therefore, I used HU as a control to show DNA damage.

First, I determined the cytotoxic effect of HU on BMDCs using AlamarBlue. BMDCs were generated as previously described. On day 7 of differentiation, BMDCs were treated with HU for 24 h with concentrations in a range of 100 μ M to 10 mM. The readout revealed that BMDCs can be treated with up to 500 μ M HU to maintain > 80 % cell viability (Figure 3.10 A). To match the cell viability with that of BMDCs treated with 10 μ M PDS (90 % survival), 100 μ M HU was used for subsequent experiments. Treatment with 100 μ M HU led to a significant increase (2.3-fold) in the protein expression of the DNA damage marker γ H2AX (Figure 3.10 B, C), further confirming previous observations that HU induces DNA DSBs. The amount of DNA damage induced with 100 μ M HU, as detected by γ H2AX protein level, was elevated compared to the amount of DNA damage induced with 10 μ M PDS (1.8-fold) (Figure 3.10 C). Further, I determined the effect of HU on the induction of apoptosis by analysing the proportion of early apoptotic cells with flow cytometry using Annexin V. With HU, the percentage of early apoptotic cells (Annexin V⁺ only) was significantly increased. On average, 13.3 % of cells were early apoptotic following HU treatment. In contrast, 6.7 % of cells were early apoptotic in vehicle-treated BMDCs and 8 % in BMDCs treated with 10 μ M PDS (Figure 3.10 D, E). Together, these data show that HU induces DNA DSBs and early apoptosis in BMDCs, even to a higher extent than PDS.

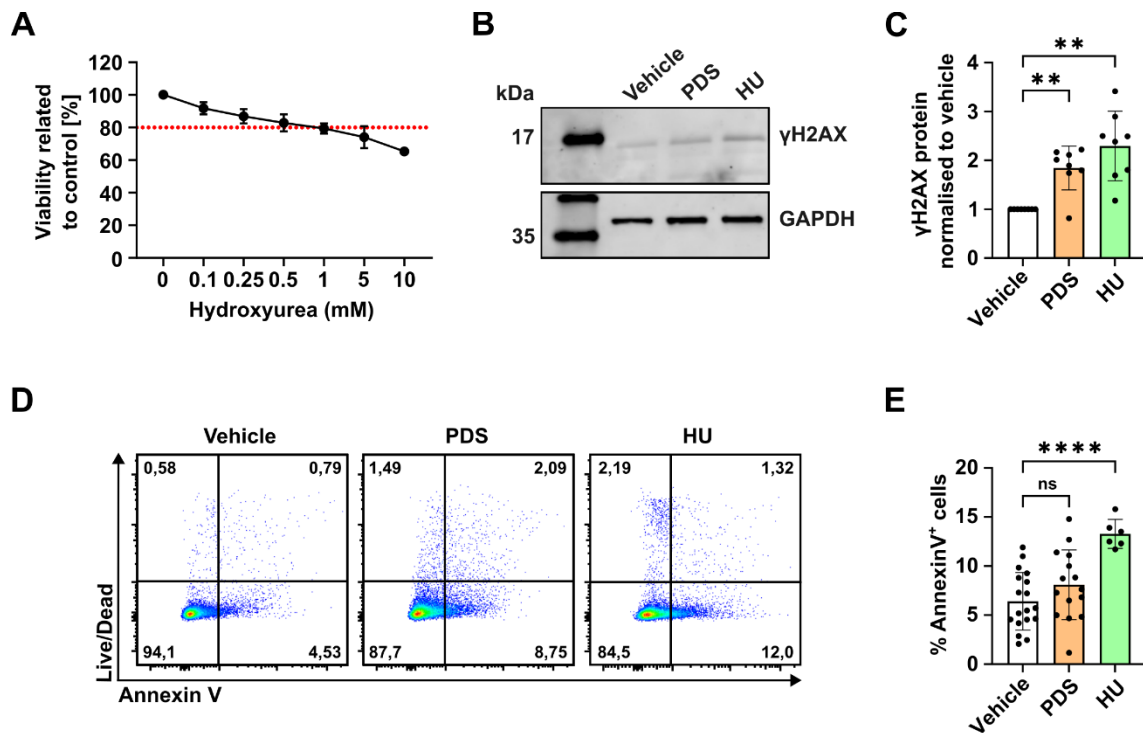


Figure 3.10 | The impact of hydroxyurea on DNA damage and apoptosis in BMDCs. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H₂O), 10 μM PDS or hydroxyurea (HU) for 24 h. A) HU cytotoxicity on BMDCs was determined using AlamarBlue. BMDCs were treated with HU concentrations ranging from 100 μM to 10 mM for 24 h. Cell viability was normalised to untreated cells (control). The red-dotted line indicates a viability threshold of 80 % in comparison to the control. Results are represented as mean ± SD based on n = 3 independent experiments. B) Representative western blot of γH₂AX (15 kDa) in vehicle-, 10 μM pyridostatin (PDS)- and 100 μM HU-treated BMDCs. GAPDH (38 kDa) was used as a loading control. C) Quantification of γH₂AX protein expression. Fold change was determined by normalising γH₂AX signal intensity over GAPDH (loading control) and further over vehicle-treated cells. Data are represented as mean ± SD based on n = 3 independent experiments. D) Representative scatter plots display the proportion of apoptotic cells in vehicle-, 10 μM PDS- and 100 μM HU-treated BMDCs. Annexin V⁺ cells are classified as early apoptotic cells, while Annexin V⁺/Live/Dead⁺ are considered late apoptotic cells. Live/Dead⁺ are interpreted as necrotic. E) Percentage of Annexin V⁺ cells within vehicle-, PDS- and HU-treated BMDCs. Data are represented as mean ± SD based on n = 3 independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (C, E). ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

To characterise the effect of DNA damage on DC activation, I examined changes in the surface expression of the DC markers CD40, CD80 and CD86 on cDC1 and cDC2 BMDCs that have been treated with 10 μ M PDS or 100 μ M HU for 24 h. As previously shown in Figure 3.3, PDS resulted in a significant increase of CD40 (cDC1: 4.7-fold, cDC2: 3.9-fold), CD80 (cDC1: 1.6-fold, cDC2: 2.1-fold) and CD86 (cDC1: 3.4-fold, cDC2: 3.9-fold) (Figure 3.11 A, B), again confirming that PDS stimulates DC activation. In contrast, HU did not significantly induce the expression of CD40 and CD86. CD40 expression was 1.5-fold increased in cDC1s and cDC2s. CD86 was just affected in cDC2 by HU, revealing a 1.7-fold elevated expression. HU treatment only caused a significant increase in CD80 on cDC1s (1.3-fold) and cDC2s (1.8-fold), however still to a lower extent compared to PDS (Figure 3.11 B). Since HU treatment induces DNA damage in BMDCs (Figure 3.10 B, C) but does not significantly affect the expression of DC activation markers, except for CD80, it can be assumed that PDS-mediated DC activation is not caused by DNA damage.

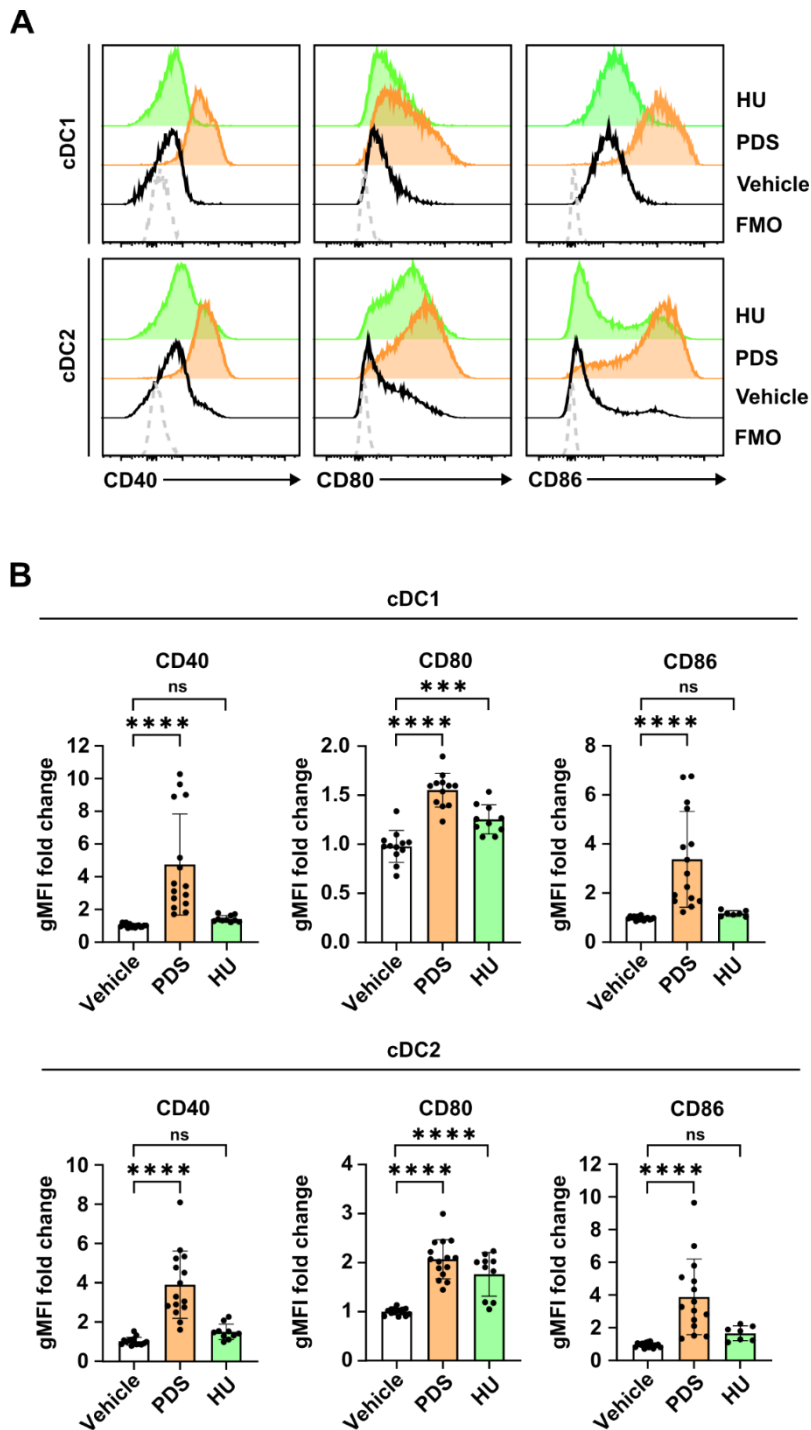


Figure 3.11 | The effect of hydroxyurea on DC activation marker expression.

Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H₂O), 10 μ M pyridostatin (PDS) or 100 μ M hydroxyurea (HU) for 24 h. Cells were stained with mAbs specific for markers of conventional dendritic cell (cDC) subsets and DC activation (CD40, CD80, CD86). DC surface marker expression was assessed by flow cytometry. A) Representative histogram showing the proportion of CD40⁺, CD80⁺ and CD86⁺ cells in vehicle-, PDS- and

HU-treated BMDCs, divided into cDC1s and cDC2s. FMO: fluorescence minus one control. B) Surface expression of the co-stimulatory molecules CD40, CD80 and CD86 on cDC1s and cDC2s following treatment with PDS or HU. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean $\pm$ SD based on at least $n = 3$ independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (B). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.8 Enhanced DC activation by PDS facilitates greater T cell priming

The main function of DCs is to induce an adaptive immune response by presenting pathogenic or aberrant self-antigens to T cells, resulting in T cell activation. T cell activation is characterised by the expression of certain T cell markers, such as CD69, CD44 and CD25, T cell proliferation and secretion of cytokines, including IFN γ , TNF α and IL-2.

In order to characterise, if PDS-mediated DC activation is sufficient to induce T cell activation, I determined phenotypical and functional changes of T cells co-cultured with vehicle- and PDS-treated BMDCs, including expression of T cell activation markers (CD69, CD44, CD25), proliferation state and cytokine production. As a positive control, T cells were co-cultured with CpG-stimulated BMDCs. In the subsequent experiments, BMDCs were co-cultured with OT-I and OT-II cells. OT-I is MHC class I-restricted CD8 $^{+}$ T cells that specifically recognise ovalbumin residues 257-264 (SIINFEKL). OT-II, in contrast, are MHC class II-restricted CD4 $^{+}$ T cells that recognise ovalbumin residues 323-339 (OVA₃₂₃₋₃₃₉).

BMDCs were generated and treated with vehicle (H₂O), 10 μ M PDS or 1 μ M CpG (positive control for DC activation) for 24 h, as previously described. On day 8 of differentiation, DCs were pulsed with 2 μ M SIINFEKL for 1 h or with 100 μ g/mL OVA₃₂₃₋₃₃₉ peptide for 4 h. Following peptide loading, 5×10^4 BMDCs were co-cultured with 1×10^5 carboxyfluorescein succinimidyl ester (CFSE)-labelled OTs for 24 h (T cell activation) or 72 h (T cell proliferation) (Figure 3.12 A). CFSE is a cell-permeable, fluorescent dye that is widely used to monitor cell proliferation

and cell tracking. As a negative control for T cell activation, OTs were co-cultured with non-peptide-loaded BMDCs (no SIINFEKL/ OVA₃₂₃₋₃₃₉), referred to as naïve OTs.

To evaluate early T cell activation, I assessed changes in surface expression of T cell markers, such as CD62L, CD44 and CD69 in OT-I and OT-II cells following 24 h of co-culture with PDS- or CpG-treated BMDCs to OT-cells co-cultured with vehicle-treated BMDCs (control). Naïve OT-I cells (no SIINFEKL) displayed a 1.8-fold higher expression of CD62L compared to OT-I cells co-cultured with SIINFEKL-loaded, vehicle-treated BMDCs (Figure 3.12 B). OT-I cells co-cultured with PDS-treated BMDCs revealed a minimal downregulation of CD62L of about 10 %, while those co-cultured with CpG-treated BMDCs significantly downregulated CD62L by about 18 % relative to the vehicle condition. Similarly, naïve OT-II cells (no OVA₃₂₃₋₃₃₉) displayed a significant increase (1.6-fold) in CD62L expression, whereas in OT-II cells co-cultured with PDS- and CpG-treated BMDCs, CD62L expression remained unchanged compared to vehicle-treated controls (Figure 3.12 C). CD44 and CD69 were not expressed on naïve OT-I cells (Figure 3.12 B). OT-I cells co-cultured with CpG-stimulated BMDCs revealed a significant upregulation of CD44 (1.8-fold) and CD69 (2.5-fold) in relation to OT-I cells co-cultured with vehicle-treated BMDCs. OT-I cells co-cultured with PDS-treated BMDCs also showed increased expression of CD44 and CD69, both about 1.3-fold, with CD69 upregulation reaching statistical significance (Figure 3.12 B). A similar expression pattern was observed on OT-II cells (Figure 3.12C). CD44 expression was significantly reduced on naïve OT-II cells, while CD69 was not expressed on those cells. Compared to vehicle-treated BMDCs, co-culture with CpG-stimulated BMDCs increased expression of CD44 (1.3-fold) and CD69 (1.9-fold) on OT-II cells. In addition, CD44 and CD69 were significantly elevated about 1.2-fold (CD44) and 1.9-fold (CD69) on OT-II cells co-cultured with PDS-treated BMDCs in relation to the vehicle condition (Figure 3.12 C). These findings demonstrate that PDS-mediated DC activation is sufficient to trigger early T cell activation.

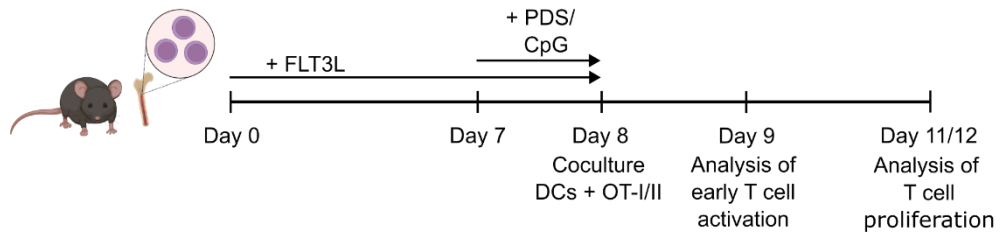
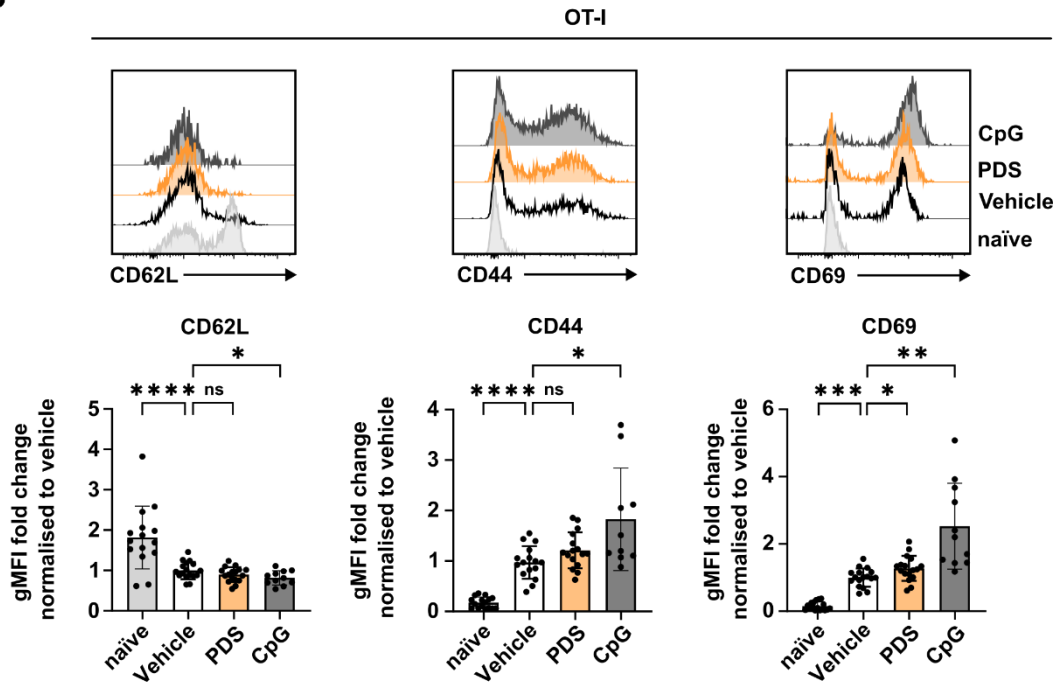
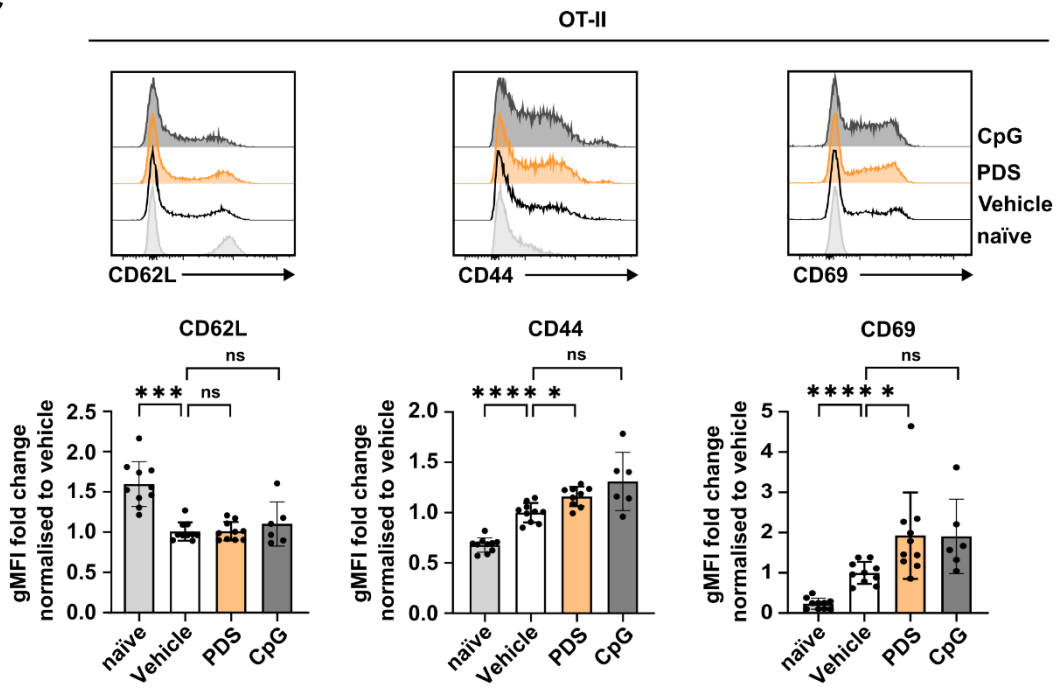
A**B****C**

Figure 3.12 | PDS-mediated DC activation promotes early T cell activation.

A) Experimental setup representing the analysis of dendritic cell (DC) functional activity on T cell induction following pyridostatin (PDS) treatment. Bone marrow-derived dendritic cells (BMDCs) were generated in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L) for seven days. Immature BMDCs were treated with vehicle (H_2O), 10 μM PDS or 1 μM CpG (positive control of DC activation) for 24 h. On day 8, BMDCs were loaded with the ovalbumin peptide SIINFEKL (OT-I-specific) or OVA₃₂₃₋₃₃₉ (OT-II-specific) and co-cultured with CFSE-labelled OT-I or OT-II T cells. After 24 h, early T cell activation was evaluated by changes in the expression of characteristic T cell activation markers. After 72h of co-culture, T cell proliferation was determined. B) Representative histograms (above) showing the distribution of CD62L, CD44 and CD69 expression on OT-I cells. Graphs (below) display the surface expression of CD62L, CD44 and CD69 on naïve OT-I (no SIINFEKL) and OT-I co-cultured with vehicle-, PDS- and CpG-stimulated, SIINFEKL-loaded BMDCs for 24 h. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to OT-I cells co-cultured with vehicle-treated BMDCs (control). Data are represented as mean $\pm$ SD based on at least $n = 4$ independent experiments. C) Representative histograms (above) showing the distribution of CD62L, CD44 and CD69 expression on OT-II cells. Graphs (below) display the surface expression of CD62L, CD44 and CD69 on naïve OT-II (no OVA₃₂₃₋₃₃₉) and OT-II co-cultured with vehicle-, PDS- and CpG-stimulated, OVA₃₂₃₋₃₃₉-loaded BMDCs for 24 h. Differences in surface expression are depicted as gMFI fold change compared to OT-II cells co-cultured with vehicle-treated BMDCs (control). Data are represented as mean $\pm$ SD based on $n = 3$ independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (B, C). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Following 72 h of co-culture, I assessed T cell activation based on changes in the surface expression of T cell markers, such as CD62L, CD44 and CD25. As expected, CD62L expression was slightly elevated in naïve OT-I cells (no SIINFEKL) (Figure 3.13 A). OT-I co-cultured with PDS-treated BMDCs showed no difference in CD62L expression compared to those co-cultured with vehicle-treated BMDCs. In contrast, CD62L expression was significantly downregulated (~49%) in OT-I co-cultured with CpG-stimulated BMDCs. A similar pattern was observed for OT-II cells. Naïve OT-II (no OVA₃₂₃₋₃₃₉) displayed a 1.9-fold increase in CD62L expression compared to peptide-loaded controls (Figure 3.13 B). As with OT-I, CD62L expression in OT-II remained unchanged after co-culture with PDS-treated BMDCs, but was significantly reduced (~23%) in response to CpG-treated BMDCs compared to vehicle. (Figure 3.13 B). CD44

and CD25 were not detectable on naïve OT-I cells (Figure 3.13 A). OT-I cells co-cultured with PDS-treated BMDCs showed no change in CD44 expression compared to vehicle-treated controls, while those co-cultured with CpG-stimulated BMDCs revealed a significant reduction in CD44 expression (~24 %). CD25 expression, however, was significantly upregulated about 1.2-fold on OT-I cells co-cultured with PDS-treated and 1.5-fold on OT-I cells co-cultured with CpG-stimulated BMDCs compared to the vehicle condition (Figure 3.13 A). A similar expression pattern was observed on OT-II cells (Figure 3.12 B). Naïve OT-II cells did not express CD44 and CD25. Co-culture with PDS-treated BMDCs did not affect CD44 expression in OT-II cells, while those co-cultured with CpG-stimulated BMDCs revealed a 20 % reduction in CD44 expression. Furthermore, CD25 expression was not significantly altered, but showed a modest increase (~1.2-fold) on both, OT-II cells co-cultured with PDS-treated and CpG-stimulated BMDCs relative to the vehicle control.

Next, I assessed the secretion of T cell-specific cytokines (IFN- γ , TNF- α , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-22) by OT-I and OT-II cells after 72 h of co-culture with vehicle- or PDS-treated BMDCs in supernatants using the cytometry-based multiplex immunoassay LEGENDplex™ (BioLegend). OT-I cells displayed an increased secretion of IFN- γ (4.3 ng/mL vs 8 ng/mL) and IL-2 (170 pg/mL vs 790 pg/mL) when co-cultured with PDS-treated BMDCs compared to vehicle-treated BMDCs (Figure 3.13 C left). For the other tested cytokines, no differences in the secretion levels were detected (data not shown). OT-II cells co-cultured with PDS-treated BMDCs revealed slightly increased levels of TNF- α (122 pg/mL vs 149 pg/mL), IL-6 (235 pg/mL vs 509 pg/mL) and IL-10 (3.6 pg/mL vs 7.6 pg/mL) in comparison to OT-II cells co-cultured with vehicle-treated BMDCs (Figure 3.13 C right). Elevated T cell-specific cytokine secretion together with increased CD25 expression indicate that PDS-mediated DC activation is sufficient to induce T cell activation, at least to a low extent.

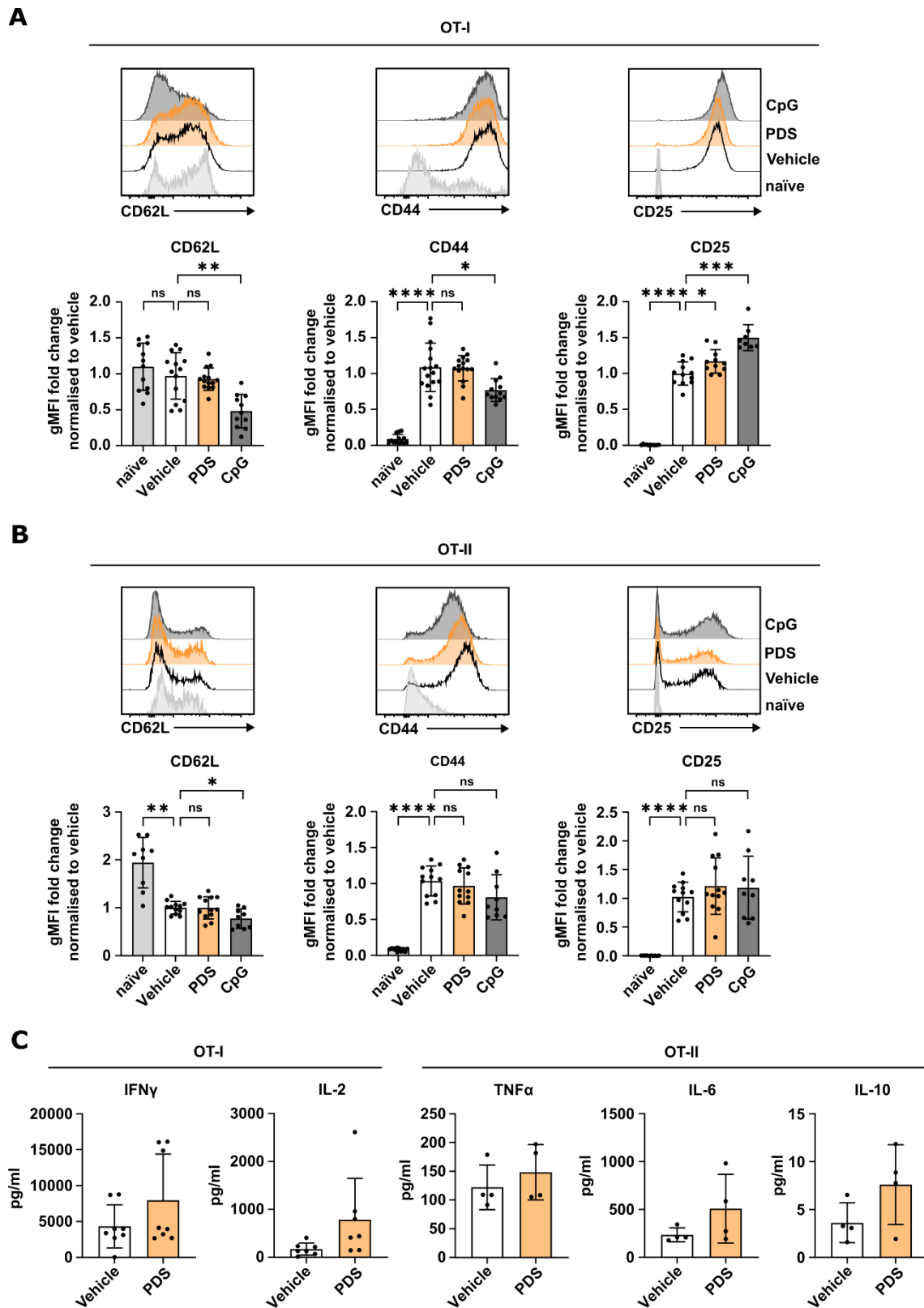


Figure 3.13 | PDS-mediated DC activation stimulates T cell activation. OT-I and OT-II cells were co-cultured with vehicle (H₂O), pyridostatin (PDS)- or CpG-stimulated bone marrow-derived dendritic cells (BMDs) loaded with ovalbumine

peptides for 72 h. A) Representative histograms (above) showing the distribution of CD62L, CD44 and CD25 expression on OT-I cells. Graphs (below) display the surface expression of CD62L, CD44 and CD25 on naïve OT-I cells (no SIINFEKL) and OT-I cells co-cultured with vehicle-, PDS- and CpG-stimulated, SIINFEKL-loaded BMDCs for 72 h. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to OT-I cells co-cultured with vehicle-treated BMDCs (control). Data are represented as mean $\pm$ SD based on $n = 5$ independent experiments. B) Representative histograms (above) showing the distribution of CD62L, CD44 and CD25 expression on OT-II cells. Graphs (below) display the surface expression of CD62L, CD44 and CD25 on naïve OT-II cells (no OVA₃₂₃₋₃₃₉) and OT-II cells co-cultured with vehicle-, PDS- and CpG-stimulated, OVA₃₂₃₋₃₃₉-loaded BMDCs for 72 h. Differences in surface expression are depicted as gMFI fold change compared to OT-II cells co-cultured with vehicle-treated BMDCs (control). Data are represented as mean $\pm$ SD based on $n = 4$ independent experiments. C) Concentrations of T cell-specific cytokines (IFN- γ , TNF- α , IL-2, IL-6, IL-10) secreted by OT-I (left) and OT-II cells (right) co-cultured with vehicle and PDS-treated BMDCs for 72 h. Cytokine concentrations were determined from the supernatant using the LEGENDplex™ Mouse Th cytokine Panel. Data are represented as mean $\pm$ SD based on $n = 3$ independent experiments. Significance was calculated using one-way ANOVA with Dunnetts' multiple comparisons test (A, B) and student's t-test (C). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Besides characterisation of changes in T cell activation marker expression and cytokine secretion, I also determined changes in the proliferation state of OT-I and OT-II cells following 72 h of co-culture with BMDCs by measuring the proliferation index. The proliferation index represents the total number of cell divisions divided by the number of cells that have undergone at least one division. The proliferation index of naïve OT-I cells and OT-II cells was significantly lower in comparison to T cells that have been co-cultured with peptide-loaded vehicle-treated BMDCs (Figure 3.14 A). This could be expected since the antigenic peptide required for MHC-TCR interaction and thus T cell activation and proliferation was missing in the naïve setting. OT-I cells and OT-II cells co-cultured with PDS-treated BMDCs and OT-II cells co-cultured with CpG-stimulated BMDCs did not show any differences in proliferation compared to vehicle. OT-I cells co-cultured with CpG-stimulated BMDCs revealed a fractional lower proliferation index of 0.89, indicating that OT-I cells co-cultured with fully activated BMDCs proliferate minimally less. When focusing on the individual division peaks, no difference in the

percentage of cells per division was observed for OT-Is co-cultured with vehicle- or PDS-treated BMDCs (Figure 3.14 B), indicating that the proliferation of OT-Is is not affected by PDS-induced DCs. However, for OT-IIs distinct differences in the percentage of cells per single division were detected. OT-IIs co-cultured with PDS-treated BMDCs revealed higher percentages of cells that have undergone three to five cell divisions (Figure 3.14 B). Conversely, a higher proportion of OT-IIs co-cultured with vehicle-treated BMDCs was either undivided or had undergone only one or two cell divisions. This indicates that OT-IIs co-cultured in the presence of PDS-treated BMDCs can divide faster.

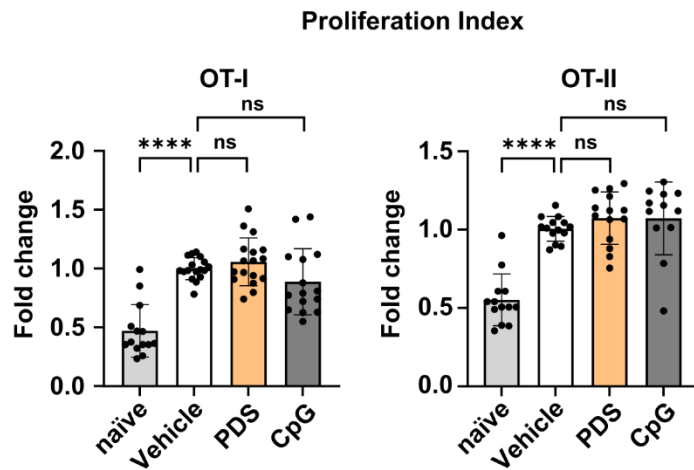
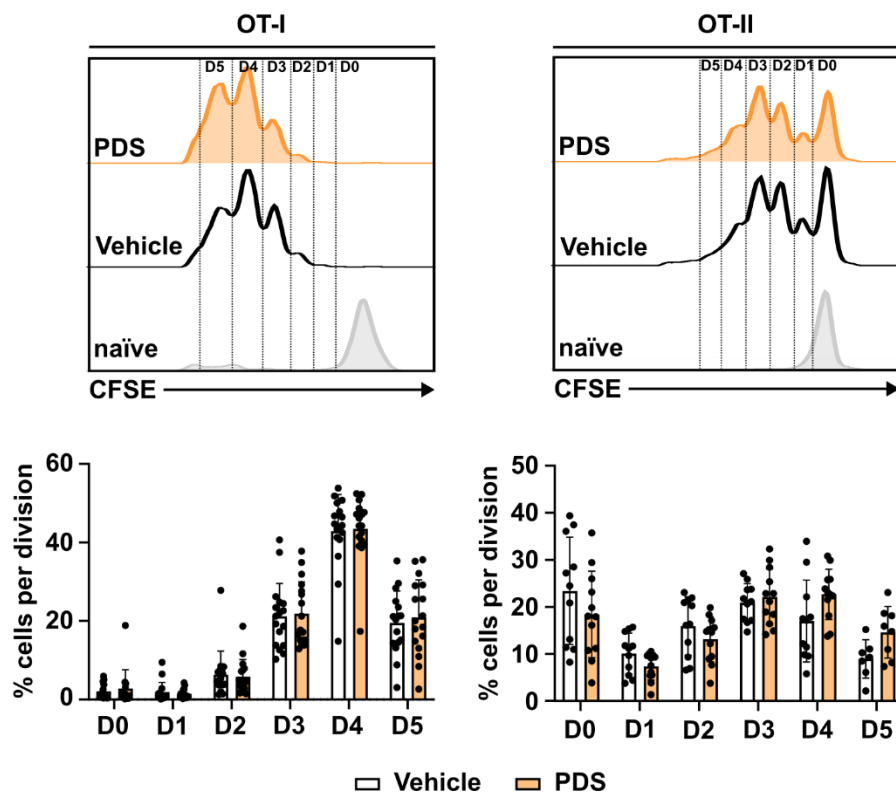
A**B**

Figure 3.14 | PDS-treated DCs stimulate proliferation of OT-II cells. OT-I and OT-II cells were co-cultured with vehicle (H₂O), pyridostatin (PDS)- or CpG-stimulated bone marrow-derived dendritic cells (BMDCs) loaded with ovalbumine peptides for 72 h. A) Proliferation index of naïve T cells (co-cultured with non-peptide-loaded BMDCs) and OT-I/II cells co-cultured with vehicle-, PDS- and CpG-treated BMDCs for 72 h. The proliferation index is depicted as fold change compared to T cells co-cultured with vehicle-treated BMDCs. Data are represented as mean $\pm$ SD based on at least $n = 4$ independent experiments. B) Representative histogram (above) showing cell proliferation by CFSE distribution

of OT-Is (left) and OT-IIs (right) co-cultured with non-pulsed BMDCs or peptide loaded vehicle- or PDS-treated BMDCs. Graphs (below) display the percentage of cells per cell division of OT-Is and OT-IIs co-cultured with vehicle- or PDS-treated BMDCs. Data are represented as mean $\pm$ SD based on at least $n = 4$ independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (A) and multiple student's t-test corrected for multiple comparisons using the Holm-Šídák method (B). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3 Discussion

G-quadruplexes are nucleic acid secondary structures that form in guanine-rich sequences of DNA and RNA. Due to their conserved localisation within specific genomic regions such as telomeres, replication origins and promoter regions, DNA G4s are suggested to have pivotal roles in biological processes such as telomere maintenance, replication and transcription (Rhodes and Lipps, 2015). So far, it is not completely understood when and how G4s form in living cells and which relevance they may have for specific cellular functions. Especially in immune cells, such as dendritic cells, G4s are barely explored. In this study, for the first time, I investigated the molecular effect of G4 formation on DC phenotype and function to gain a better understanding of the relevance of G4 structures in cellular pathways important for DC regulation.

3.3.1 G4 stabilisation by PDS induces DC maturation and activation

DCs are antigen-presenting cells that patrol in peripheral tissues to recognise danger signals such as DAMPs (self-antigens) and PAMPs (e.g. LPS, CpG), which will be processed into antigenic peptides and presented in lymph nodes to CD4⁺ and CD8⁺ T cells, resulting in the induction of an adaptive immune response. Antigen uptake and peptide processing initiate the DC activation and maturation process, which is associated with several phenotypical and functional changes, including increased expression of MHC class I and class II molecules, upregulation of co-stimulatory molecules (e.g. CD40, CD80, CD86), increased production of immunomodulatory cytokines, enhanced migration and loss in antigen capture (Banchereau et al., 2000; Reis E Sousa, 2006).

CpG oligonucleotide is a well-known adjuvant used to activate DCs *in vitro* and *in vivo* by triggering TLR9 expressed in endosomes of DCs (Jin et al., 2017; Sparwasser et al., 1998). In agreement with these studies, administration of CpG *in vitro* resulted in intensive upregulation of co-stimulatory molecules and MHC molecules on BMDCs, confirming DC activation (Figure 3.4 A, B). To investigate the impact of G4 formation on DC activation and maturation, I stabilised G4

structures using the G4 ligand PDS, which has already been reported to stabilise G4s in various cell types and organisms (Lejault et al., 2020; Lv and Zhang, 2023; Rodriguez et al., 2012; Zou et al., 2021). Accordingly, PDS also stabilises G4s in BMDCs (Figure 3.1 C, D). PDS-mediated G4 stabilisation resulted in a significant upregulation of CD40, CD80, CD86, MHC I and MHC II on BMDCs, demonstrating that PDS triggers DC activation (Figure 3.4 A, B). However, the surface expression levels were not as high as in CpG-stimulated (fully activated) DCs, showing that DC activation following G4 stabilisation is not as strong as with CpG. Similarly, only a modest effect of PDS on human MoDCs was observed (Figure 3.5 C). According to viability testing, I used a concentration of 10 μ M PDS for all *in vitro* experiments. Increasing concentrations of PDS application might enhance the effect on the expression of co-stimulatory molecules. However, due to PDS-mediated cytotoxicity, a maximum concentration of 15 μ M PDS could be used on BMDCs to avoid possible effects based on apoptotic or necrotic mechanisms (Figure 3.1 B). Expression of some DC markers, such as CD86 and MHC II was increased to a higher extent on cDC2s compared to cDC1s following PDS administration, showing that the effect of G4 stabilisation causes a stronger response in cDC2s (Figure 3.4 A, B). A previous study demonstrated that co-stimulatory molecules are differentially expressed on different DC subsets (Carenza et al., 2019). In this study, they observed that cDC1s reveal higher CD40 but lower CD86 expression than cDC2s in basal conditions, nevertheless, differences in CD86 expression were even maintained upon TLR-mediated stimulation. Although evident explanations for these differences are still missing, the differential roles of CD40 and CD86 in immune activation, suggest a link between the differential expression of these molecules and the functional specialisation of DC subsets (Carenza et al., 2019). My research suggests that such differences may at least in part be regulated by G4s.

Besides DC activation, PDS also triggers DC maturation. Events associated with DC maturation include enhanced expression of the chemokine receptor CCR7, induced migration and reduced antigen-capture activity (Banchereau et al., 2000). In line with this, PDS treatment of BMDCs caused an enrichment of CCR7 on the surface, enhanced migration towards a CCL19 gradient and a marginal

reduction in antigen uptake (Figure 3.6). In contrast to Figure 3.4, where the entire population upregulated DC activation markers, only a subset of cells showed increased CCR7 expression in response to PDS (Figure 3.6 A). A possible explanation for the observed differences in population shifts may be that in Figure 3.4, the parameters were examined in distinct DC subsets (cDC1, cDC2), whereas CCR7 expression was assessed in the whole DC population (CD11c⁺MHCII⁺ cells) (Figure 3.6 A, B). Similar to DC activation, G4 stabilisation does not induce DC maturation as strongly as observed with CpG stimulation. This leads to the suggestion that CpG stimulation and G4 stabilisation induce DC maturation and activation through different pathways. CpG interaction with TLR9 leads to the activation of transcription factors, like NFκB, JNK, p38 and ERK1/2, resulting in DC maturation and production of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12) (Wagner, 2001). In contrast, PDS stabilises G4 structures throughout the whole genome resulting in the activation of several pathways. G4 structures in gene promoter regions often serve as a transcription factor binding hub (Lago et al., 2021; Spiegel et al., 2021), leading to increased gene expression. However, a connection between G4 structures and the TF NFκB has not been reported so far.

3.3.2 G4 stabilisation leads to transcriptional reprogramming in DCs

A recent study has shown that PDS induces transcriptional changes in neurons (Escarcega et al., 2023). In agreement with this, PDS also induces transcriptional reprogramming in BMDCs. FLT3L-derived BMDC cultures are quite heterogeneous and are composed of all functional DC subsets including cDCs (60-70 %) and pDCs (15-20 %) (Naik et al., 2005). PDS-induced differentially expressed genes could be assigned to various biological processes identified by gene ontology analysis. The biological process revealing the strongest enrichment was “response to virus” (Figure 3.9 B). Plasmacytoid DCs are the main players in cellular anti-viral defence due to their capacity to produce high amounts of Type I IFN (Cella et al., 2000; Siegal et al., 1999). Several ISGs associated with an anti-viral defence response, such as Mx1, Mx2, Ifih1 (Mda5),

Ifit1/2, IRF7, Rsad2, and RTP4 were identified to be significantly upregulated upon PDS treatment (Figure 3.9 A). These genes mainly take part in viral sensing and inhibition of viral replication through various effector functions (Daffis et al., 2008; Haller et al., 1995; Honda et al., 2005; Kato et al., 2006; Pichlmair et al., 2011; Saitoh et al., 2011; Schmid et al., 2010; Wang et al., 2007). These data show that PDS stimulates an anti-viral immune response in DCs, presumably in pDCs, by upregulating the expression of the mentioned anti-viral genes. Anti-viral genes are usually induced in response to viral sensing (Chathuranga et al., 2021). Among others, IRF7 is one TF involved in the induction of ISGs, even in the absence of type-I IFN signalling (Schmid et al., 2010). Genome-wide mapping of DNA G4 structures identified a potential G4 motif in the IRF7 promoter under PDS stabilising conditions (Marsico et al., 2019). These findings suggest that PDS-mediated G4 stabilization in the promoter region of *IRF7* may contribute to increased *IRF7* expression, which could in turn support the induction of antiviral-associated ISG expression in pDCs within the BMDC culture. Future experiments should address this hypothesis, including chromatin immunoprecipitation (ChIP) and IRF7 pulldown assays. Importantly, molecules necessary for viral sensing and type-I IFN production such as MDA-5 and IRF7 are not exclusive of pDCs. A previous study revealed that also cDC2s can sense viral infection and produce IFN α (Luber et al., 2010). Due to the existence of both, pDCs and cDCs in BMDC populations, single-cell sequencing could provide further insights into which genes were differentially expressed in the respective DC subsets.

Other biological processes identified include “response to external stimulus”, “response to stress”, “innate immune response”, “cytokine production”, “cell activation” and “lymphocyte activation” (Figure 3.9 B). Although PDS is cell permeable, the induction of genes associated with a “response to external stimulus” leads to the suggestion that PDS might be recognised by PRRs of DCs as a foreign molecule, inducing a signalling cascade resulting in the activation of genes underlying this response. However, no interaction of PDS with other structures than G4s has been reported so far. On the other hand, as PDS targets various G4 structures in the genome (Ray et al., 2020), these genes could also be randomly affected and induced by PDS-mediated G4 stabilisation within the

genome. Although a concentration of 10 μ M PDS reached a survival rate of 90 %, some cells were dying due to PDS cytotoxicity. Hence, free DNA from dying cells within the BMDC culture could also be recognised by nucleic-acid sensors in DCs, inducing a response to an external stimulus.

Furthermore, PDS-mediated G4 stabilisation in DCs induced a program of genes linked to the “response to stress”. A “cellular response to stress” (as defined by the GO term 0006950) is triggered by any change in the state or activity of a cell as a result of a stimulus. As previously shown, PDS causes a change in DC state and activity by triggering DC maturation and activation, hence initiating stress. Furthermore, PDS-mediated G4 stabilisation has been reported to cause replication and transcription-dependent stress due to DNA DSB formation (Rodriguez et al., 2012), resulting in the reprogramming of genes linked to DNA repair. However, no DEGs associated with DNA repair were identified within the cluster of genes assigned to the “response to stress”, concluding that DNA damage might not be a prominent cause for the induction of stress response. Furthermore, similar to the “response to external stimulus”, free DNA due to PDS-induced cell death within the BMDC culture could cause a “response to stress” in DCs. Stress granules are defined as membrane-less cytoplasmic compartments composed of RNA-binding proteins, stalled mRNA and various translation initiation factors that assemble in response to cellular stress (Jain et al., 2016). Recently, it has been shown that PDS induces the assembly of stress granules (SGs) in a non-small cell lung cancer cell line, which might contain DNA (Iida et al., 2022). However, the molecular mechanism of PDS-induced SG assembly is still under investigation. Whether PDS leads to SG assembly in DCs could be identified by colocalisation of BG4 with SG markers such as T cell-restricted intracellular antigen-1 (TIA-1) and GTPase-activating protein-binding protein 1 (G3BP1) using immunofluorescent staining. Nevertheless, these findings highlight several options by which PDS can trigger a response to stress in DCs.

Furthermore, analysis of PDS-induced transcriptional reprogramming identified that PDS-mediated DC activation, as previously observed through upregulation of co-stimulatory molecules on the cell surface, is triggered on the transcriptional

level by stimulating the expression of genes affiliated with DC activation (Figure 3.9 C). PDS-induced DEGs associated with DC activation included chemokines (CXCL2, CXCL9, CCL3, CCL4, CCL5), cytokines (IL-12b, IL-6, IL-1 β), co-stimulatory molecules/surface markers (CD40, CD80, CD83, CD86, CCR7) and regulatory proteins (STAT1, Socs2), which have major effector functions in DC maturation, migration, chemoattraction, antigen presentation as well as T cell activation and differentiation (Ashour et al., 2020; Fujii et al., 2004; Hu et al., 2012; Jackson et al., 2004; Ohl et al., 2004; Ohtani et al., 2009; Rawat et al., 2023; Rincón et al., 1997). These findings were further supported by gene ontology analysis identifying biological processes linked to DC activation and maturation, such as “innate immune response”, “cytokine production”, “cell activation” and “lymphocyte activation” (Figure 3.9 B).

With these findings, I could show that phenotypical changes in DCs triggered by PDS-mediated G4 stabilisation are based on transcriptional reprogramming, resulting in the induction of several stimulatory pathways. However, it remains unclear, how G4 stabilisation leads to the transcriptional changes. Depending on the location of the G4 motif within the gene, G4 formation can differentially induce gene transcription. G4 formation within the gene body on the non-template strand facilitates transcription by keeping the DNA strands in a single-stranded confirmation (Agarwal et al., 2014). Moreover, G4s are overrepresented in promoter regions where they have been reported to positively or negatively affect gene transcription (Huppert and Balasubramanian, 2007; Lago et al., 2021). Genome-wide mapping of DNA-G4 structures revealed G4 motifs in the promoter regions of several genes connected to DC activation and maturation (CD40, CD80, CD86, CCR7, IL-6, IL-12b, CD83, STAT1, IL-1 β) under PDS-stabilising conditions in the mouse genome (Marsico et al., 2019). This suggests that PDS-induced gene expression in DCs might be due to G4 formation in the promoters of the respective genes. G4 formation in gene promoters has been shown to stimulate transcription through the recruitment of TFs (Lago et al., 2021; Spiegel et al., 2021). Future experiments including G4-ChIP to identify G4 formation in the particular gene promoters and Co-IPs to detect G4 interaction with TFs in the genes associated with DC activation need to be performed. Spiegel et al. (2021)

showed that PDS competes with TF binding to G4 DNA. This could be a possible explanation why PDS does not result in as strong upregulation of co-stimulatory molecules on the DC surface as observed with CpG stimulation, due to reduced binding of the TF to the G4 structure in the respective gene promoter.

Transcriptome-wide analysis (RNA-Seq) revealed that expression of genes encoding cytokines such as IL-12b, IL-6 and IL-1 β , which play a major role in T cell differentiation (Heink et al., 2017; Heufler et al., 1996; Revu et al., 2018) were significantly upregulated in DCs upon PDS treatment (Figure 3.9 C). However, no differences in cytokine secretion levels were detected compared to unstimulated DCs. Changes in gene expression do not automatically imply changes on the protein level. A possible explanation could be that the mRNA stability/cytokine expression was post-transcriptionally regulated by miRNAs or RNA-binding molecules (Asirvatham et al., 2009; Kovarik et al., 2017; Palanisamy et al., 2012). Furthermore, cytokine levels were measured within the supernatant. It could be possible that cytokines were produced but have been degraded within the supernatant due to short half-life or freeze and thawing conditions or were not secreted by the DCs due to only partial maturation following PDS stimulation (G et al., 2014; Liu et al., 2021; Jing Wang et al., 2017). Measuring cytokine levels in cell lysate or staining for cytokines intracellularly could give further insights. Additionally, the cytokine could have been present but below the detection threshold of the Legendplex Assay (~1-10 pg/mL) (LEGENDplex – Technical Data Sheet, BioLegend (2023)). Furthermore, it could also be possible that the cytokine secretion peaks earlier or later than 24 h (Langenkamp et al., 2000), hence measuring cytokine levels at different timepoints (e.g. 6 h, 12 h, 18 h, 24 h, 48 h) could be an additional option to test.

3.3.3 G4 ligands such as Braco-19, CX-5461 and TMPyP4 differentially affect the DC phenotype

In this study, I demonstrated that the PDS-mediated effect on DCs is likely due to G4 stabilisation by testing the effect of other G4 ligands, such as BRACO-19, CX-

5461 and TMPyP4, on changes in the surface expression of DC co-stimulatory molecules (Figure 3.8). Here, it needs to be mentioned that the results gained with PDS are slightly different to those shown in Figure 3.4, which can be explained by the fact that these two experiments were set up in different laboratories (Melbourne and Bonn) and two different devices for flow cytometry analysis have been used. In line with PDS, BRACO-19 induced upregulation of co-stimulatory molecules (CD40, CD80, CD86) on DCs, confirming the effect of G4 stabilisation on triggering DC activation. Interestingly, CX-5461 and TMPyP4 only affected the expression of single molecules, but not all activation markers as observed for PDS and BRACO-19. A possible explanation could be that G4 ligands have different preferences for G4 motifs. Importantly, TMPyP4 and CX-5461 were initially developed for applications other than G4 stabilisation, hence G4 stabilisation was not their primary function. TMPyP4 was previously used as a photosensitizer in photodynamic therapy (Villanueva et al., 1994) and CX-5461 was initially developed as a RNA polymerase I inhibitor used in haematological malignancies (Khot et al., 2019). However, recent studies exposed that the mechanism underlying the therapeutic efficacy of CX-5461 included G4 stabilisation and the inhibition of topoisomerase II (Bossart et al., 2021; Bruno et al., 2020; Pan et al., 2021). Since TMPyP4 and CX-5461 have not been developed to specifically bind to G4 structures, it could be possible that they only target selective G4s. This hypothesis is supported by the fact that G4 ligands reveal distinct binding affinities towards G4s, dependent on G4 topology (parallel, anti-parallel, hybrid) and structure (number of G-tracts, loop length, capping structure), allowing specific recognition by G4 binding molecules (Chen et al., 2022; Machireddy et al., 2019; Mita et al., 2006). Furthermore, G4 ligands bind to G4 structures using different binding modes (top stacking, end stacking, bottom stacking, groove binding), which may affect G4 stabilisation and the effect on cellular processes. Molecular dynamics (MD) simulations suggest that CX-5461 and TMPyP4 primarily bind to the top of G4 structures while BRACO-19 might use various binding modes dependent on G4 topology (Machireddy et al., 2019; Paradis et al., 2022; Sullivan et al., 2020). Moreover, it needs to be considered that ligand-G4 interactions can additionally be interfered with by specific G4

interactions with proteins, epigenetic modifications in promoters or single nucleotide polymorphisms. Additionally, TMPyP4 reveals only low G4 specificity as it also binds to duplex DNA, resulting in off-target effects. Besides G4 stabilisation, several studies reported a G4-unfolding activity of TMPyP4, which could be another possible explanation for the variation in co-stimulatory molecule regulation, suggesting that TMPyP4 might have a stabilising function on G4s in CD40 and MHC II gene promoters and an unfolding activity on CD80 and CD86 G4s. Future experiments including G4-pulldown with biotinylated TMPyP4 to confirm the binding of TMPyP4 to the respective promoter G4s are of interest to clarify the controversial function of TMPyP4 on co-stimulatory molecule regulation. Nevertheless, the dual function of TMPyP4 on G4 stabilisation and unfolding might be a possible reason for the unchanged G4 levels observed by BG4 immunofluorescence microscopy (Figure 3.7 B, C), as both effects might neutralise each other. Another possible explanation could be the off-target effect due to its low G4 specificity or its function as a photosensitizer, as it might indirectly interfere with the BG4 signal intensity by quenching or photobleaching the fluorescent signal. Taken together, the use of different G4 ligands provides evidence for a role of G4 stabilisation in regulating DC surface molecule expression. Nevertheless, several G4 ligands have different G4 structure preferences, which may result in the induction of different cellular pathways and distinct phenotypical profiles.

3.3.4 PDS-mediated cellular changes in DCs are not caused by DNA damage

Previously, PDS has been reported to be cytotoxic to cell lines by inducing DNA damage and promoting cell cycle arrest (Bossaert et al., 2021; Hou et al., 2022; Rodriguez et al., 2012). In agreement with this, I found that PDS treatment induces DNA damage in BMDCs and causes a minor population of cells to shift towards the G2 phase (Figure 3.2 A-D), an indication of required DNA repair. PDS-mediated DNA damage induced through G4 stabilisation can result in cellular changes, such as altered transcription. Rodriguez et al. (2012) reported

that G4 stabilisation by PDS leads to replication- and transcription-dependent DNA damage, resulting in genome instability and altered expression of damaged genes. Here, I demonstrated that cellular changes in DCs observed upon PDS treatment, such as transcriptional reprogramming and upregulation of cell surface marker expression associated with DC activation are not caused by PDS-induced DNA damage and the subsequent DNA repair response by treating cells with hydroxyurea, a DNA damaging agent. HU is a genotoxic agent that affects the activity of the ribonucleotide reductase, resulting in a depleted dNTP pool and replication stress. Prolonged HU exposure or higher dosages cause replication fork stalling, resulting in genome instability and DNA DSB formation (Young and Hodas, 1964). Similar to PDS, HU induced DNA damage in BMDCs, even to a stronger extent. In addition, HU also induced apoptosis in BMDCs which is not the case for PDS (Figure 3.10). This could be due to the prolonged exposure of the cells to HU, which leads to the accumulation of DNA damage and oxidative stress, causing cell death (Huang et al., 2016; Marchetti et al., 2006). In contrast to PDS, HU did not impact the expression of co-stimulatory molecules on BMDCs, except for CD80 (Figure 3.11). A possible explanation could be that CD80 expression might be increased by HU-induced ROS (Marchiori et al., 2019), indicating a DNA damage-mediated response. However, a relationship between PDS and ROS has not been reported so far. Based on the fact that both agents, PDS and HU, induce DNA damage in BMDCs, but HU does not affect the expression of the co-stimulatory molecules (except for CD80), it can be assumed that cellular changes upon PDS treatment are not caused or induced by DNA damage. This conclusion is further supported by the fact that PDS did not induce any changes in gene expression levels of DNA repair proteins involved in DSB repair (Table 18), an essential aspect of an efficient DNA damage response (DDR) (Karakaidos et al., 2020). A possible explanation could be that the DNA damage induced by PDS is not severe and can be repaired by the basal presence of DNA repair proteins (Carlomagno et al., 2000; Tomascik-Cheeseman et al., 2004). This hypothesis is further supported by the minor percentage of cells, shifted towards the G2 phase upon PDS treatment. Future experiments, including a western blot to detect the activation of DNA repair genes by protein

phosphorylation, could give further insights into the induction of DDR in PDS-treated BMDCs.

3.3.5 PDS-mediated DC stimulation is sufficient to induce T cell priming

The main function of DCs is to induce an adaptive immune response by presenting antigens to T cells, resulting in T cell activation. Here, I demonstrated that PDS stimulation of DCs induces DCs' capacity of T cell priming, especially of CD4⁺ (OT-II) T cells (Figure 3.12). This is consistent with the stronger effect of PDS on cDC2 stimulation compared to cDC1. Additionally, cDC2s are about 5 to 10-fold more abundant than cDC1s in BMDC populations, hence more cells are present to trigger a stronger OT-II activation compared to OT-I. Previous studies demonstrated that activation of stress response pathways can program DCs to efficiently stimulate T cells (Osorio et al., 2014; Ravindran et al., 2014). Transcriptome analysis revealed that PDS administration triggers a response to stress in DCs, thus DC-mediated T cell stimulation might be referable to a PDS-induced stress response in DCs. The effect of PDS on DCs' ability to stimulate T cell activation is further supported by the fact that CD4⁺ T cells co-cultured with PDS-pretreated BMDCs proliferate faster compared to T cells co-cultured with vehicle-treated BMDCs (Figure 3.14). Interestingly, CD25 (late T cell activation marker) was only slightly upregulated on OT-IIs co-cultured with PDS-pretreated BMDCs (Figure 3.13B). Proliferation analysis revealed that OT-IIs generally proliferate slower compared to OT-Is (Figure 3.14 B). Hence, measuring T cell activation of OT-IIs later than 72 h might result in higher expression changes of CD25.

No difference was observed in the proliferation of CD8⁺ (OT-I) T cells co-cultured with vehicle- or PDS-pretreated BMDCs. However, at the time point of proliferation measurement (after 72 h of co-culture), almost all OT-I cells had undergone at least three cell divisions, showing that these cells proliferate quite fast. This could also be a possible reason why only a minimal effect was observed on the early activation of OT-I cells. CD69 (early T cell activation marker) surface

expression peaks at 16 h after activation (Wu et al., 2019). However, early activation of T cells was determined 24 h after co-culture with BMDCs, thus expression of CD69 could already be declined again. Therefore, measuring activation and OT-I proliferation at earlier time points might clarify if PDS stimulation of DCs also enhances the ability of cDC1s to stimulate CD8⁺ T cells. This would further be in line with the significantly increased expression of CD25 observed on CD8⁺ T cells co-cultured with PDS-treated BMDCs (Figure 3.13 A). CD25 has also been reported to be upregulated on CD8⁺ T cells following activation (Shatrova et al., 2016). Furthermore, the transgenic TCR of OT-I cells is highly sensitive towards the ovalbumin peptide SIINFEKL, which induces full T cell activation independent of any need for costimulation (Denton et al., 2011; Zehn et al., 2009). Hence, differences in DC activation induced by PDS might be irrelevant for OT-I activation and proliferation, if the affinity threshold for naïve CD8⁺ T cell activation is exceeded by the stimulus. However, this might be different for endogenous CD8⁺ T cells with a natural TCR repertoire, which are dependent on three signals (MHC II/TCR interaction, co-stimulation and cytokines) to become fully activated (Murphy and Weaver, 2017). Therefore, I assume that enhanced DC activation mediated by PDS-induced G4 stabilisation would lead to increased activation of endogenous T cells. This hypothesis could be addressed in a mixed lymphocyte reaction by co-culturing splenic T cells with PDS-pretreated or untreated BMDCs loaded with a pool of antigens.

T cell activation was further supported by the observation that T cells co-cultured with PDS-treated BMDCs secreted higher levels of T cell-specific cytokines. However, none of these changes reached statistical significance due to the high variability of cytokine concentrations within the individual samples (Figure 3.13 C). Moreover, for some samples, cytokine levels couldn't be detected, which could be explained by a failed proliferation, thus no T cell activation. Therefore, increased sample size could improve the statement about cytokine secretion and the T cell activation state. Furthermore, using BMDCs at earlier time points (Day 6 or 7 of differentiation) might enhance T cell activation in co-culture experiments, as longer culture times may impair BMDC activity.

In conclusion, these findings highlight that PDS-driven G4 stabilisation leads to transcriptional reprogramming in DCs, causing the stimulation of several pathways associated with DC activation and maturation, resulting in enhanced DC function of T cell priming.

4. Results – G-quadruplex stabilisation in dendritic cells as immunotherapeutic target in B16OVA tumour and Graft-vs-host-disease mouse model

4.1 Introduction

In the last decades, different promising anti-cancer therapeutic strategies have been developed. Amongst others, current immunotherapeutic strategies focus on blocking immune checkpoint molecules on the surface of tumour and immune cells using monoclonal antibodies to enhance anti-tumour immune responses. However, genetic and epigenetic abnormalities in tumour cells as well as the presence of tumour-supporting oncogenes highlight the need for targeted therapies on the molecular level. G4 stabilisation by G4 ligands is currently discussed as an anti-tumour treatment strategy (Carvalho et al., 2020; Cimino-Reale et al., 2017). Several G4 ligands were shown to have anti-cancer properties, including telomestatin, RHPS4, Braco-19 and PDS (Burger et al., 2005; Groelly et al., 2022; Salvati et al., 2007; Tauchi et al., 2006). Three different G4-induced mechanisms that impair tumour growth have been identified so far. These mechanisms rely on G4-mediated telomerase inhibition, reduction of oncogene expression and induction of genome instability leading to apoptosis (Calabrese et al., 2018; Felsenstein et al., 2016; Gowan et al., 2002; Groelly et al., 2022; Wang et al., 2019b). All these approaches focus on a direct impact on cancer cells, aiming to kill tumour cells and thus slow down tumour progression. However, besides directly targeting tumour cells, other therapeutic approaches using G4 stabilisation could also focus on the initiation of anti-tumour immune responses. DCs play a crucial role in inducing an anti-tumour immune response by cross-presenting tumour antigens via MHC I to CTLs (Roberts et al., 2016). However, the tumour microenvironment is composed of suppressive factors, including immunosuppressive cell populations, metabolites and anti-inflammatory cytokines that impede DC activation, maturation and thus their function to initiate an anti-tumour response (Xiao et al., 2023). Based on my previous findings *in vitro*, I assume that G4 stabilisation *in vivo* might modulate DCs in the TME towards a more activated state. DC activation within the TME in

turn might lead to improved T cell activation and finally result in improved induction of effective anti-tumour immunity.

In this study, I assessed the impact of G4 stabilisation on DCs within the TME in two different tumour mouse models, B16OVA melanoma and lung metastasis. The impact of G4 stabilisation on DC activation in the tumour was determined by measuring changes in the surface expression of the co-stimulatory surface proteins CD40, CD80 and CD86.

Acute GvHD is a severe complication that can occur after allogeneic stem cell transplantation. aGvHD is characterised by the onset of symptoms within 100 days following allo-HCT. Allo-HCT aims to replace patient's stem cells with those from an HLA-matched donor to treat haematological malignancies by eradicating leukaemic cells recognised by donor T cells (GvL effect) (Copelan, 2006). In aGvHD, immunocompetent donor T cells recognise the host as foreign and induce an immune response resulting in the attack of host tissues and organs (Zeiser and Blazar, 2017). aGvHD is initiated by an allo-HCT conditioning regimen (e.g. chemotherapy and/or radiotherapy), which leads to host tissue damage, particularly damage to the intestinal mucosa. Tissue damage results in the release of DAMPs and PAMPs that are recognised by host APCs (e.g. DCs, macrophages and B cells), which further present the antigens to alloreactive donor T cells (Malard et al., 2023). Due to the HLA mismatch between host APC and donor T cell, the donor T cell recognises the presented host antigen as foreign and induces an immune response against the respective target tissue, referred to as aGvHD (Zeiser and Blazar, 2017). Therapeutic approaches for aGvHD include prophylactic treatment options that mainly focus on T cell immunity (e.g. T cell depletion, inhibition of T cell activation and proliferation) (Storb et al., 1989; Wagner et al., 2005; Zeiser et al., 2020). However, given the fact that DCs play a central role in the initiation of the disease, regulation of DC activation or reprogramming of DCs into a tolerogenic phenotype could be considered as an additional therapeutic strategy to prevent the presentation of host antigens to alloreactive donor T cells. G4 structures have been reported to alter chromatin organisation and subsequent gene expression, resulting in

transcriptional reprogramming of cells (Lawler et al., 2023). In addition, I have previously shown that G4 stabilisation leads to transcriptional reprogramming of BMDCs *in vitro* (Figure 3.9), associated with DC activation. Therefore, G4 stabilisation represents a promising therapeutic strategy to reprogram the DC phenotype.

Here, I assessed the impact of G4 stabilisation on DCs in aGvHD in an MHC major-mismatched aGvHD mouse model. The effect of G4 stabilisation on DC activation in aGvHD was determined by measuring changes in the surface expression of the co-stimulatory surface proteins CD40, CD80 and CD86. In addition, the impact of G4 stabilisation on the overall progression of aGvHD was evaluated by monitoring survival and occurrence of GvHD-specific symptoms such as weight loss, (bloody) diarrhoea, skin rash and fur loss in GvHD-induced mice. According to my previous findings *in vitro*, I assume that G4 stabilisation, in contrast to the tumour setting, might worsen the progression of acute GvHD in mice.

Taken together, in this chapter I aimed to characterise the use of G-quadruplex stabilisation in DCs as an immunotherapeutic target in two settings, B16OVA tumour and aGvHD. In both settings, DCs play a critical role. However, their functional outcome in tumour and GvHD is like a double-edged sword.

4.2 Results

4.2.1 Intratumoural injection of PDS induces DC activation in B16OVA melanoma

The impact of G4 stabilisation on DCs in the TME was characterised using the B16OVA melanoma mouse model. The mouse model was used due to its potential to specifically investigate anti-tumour immune responses. B16OVA cells express the immunogen ovalbumin, which is recognised by DCs as a tumour antigen. DCs process ovalbumin into the antigen SIINFEKL and present it to T cells, inducing an anti-tumour immune response.

Mice were injected with 5×10^5 B16OVA cells subcutaneously on day 0. Once the tumour reached a size of $\geq 40 \text{ mm}^3$, 0.5 mg PDS was injected intratumourally (i.t.). 48 h after treatment, the tumour was harvested and DCs within the tumour were characterised by flow cytometry (Figure 4.1 A). DCs *in vivo* are characterised based on CD45⁺F4/80⁻CD11c⁺MHCII⁺ expression. cDC1 and cDC2 subtypes are distinguished by CD8 α (cDC1) and CD11b (cDC2) (Figure 4.2 B).

I characterised the impact of G4 stabilisation by PDS on DCs within the tumour by assessing changes in the surface expression of the co-stimulatory molecules CD40, CD80 and CD86. Surface expression of CD40, CD80 and CD86 was significantly increased on cDC1s (CD40: 1.7-fold, CD80: 2.2-fold, CD86: 3-fold) and cDC2s (CD80: 1.7-fold, CD86: 2.4-fold) in tumours treated with PDS (Figure 4.1 C). This shows that intratumoural injection of PDS induces DC activation in the tumour. However, the proportion of DCs, indicated by CD11c⁺MHCII⁺ cells, was significantly reduced from 16.5 % to 8 % in tumours injected with PDS (Figure 4.1 D). Interestingly, focusing on the proportional composition of the respective DC subtypes revealed that the cDC1 population was significantly decreased (5.7 % to 2 %) in tumours injected with PDS, while the cDC2 population was significantly increased (67 % to 82 %) in comparison to PBS-treated tumours (vehicle) (Figure 4.1 E, F).

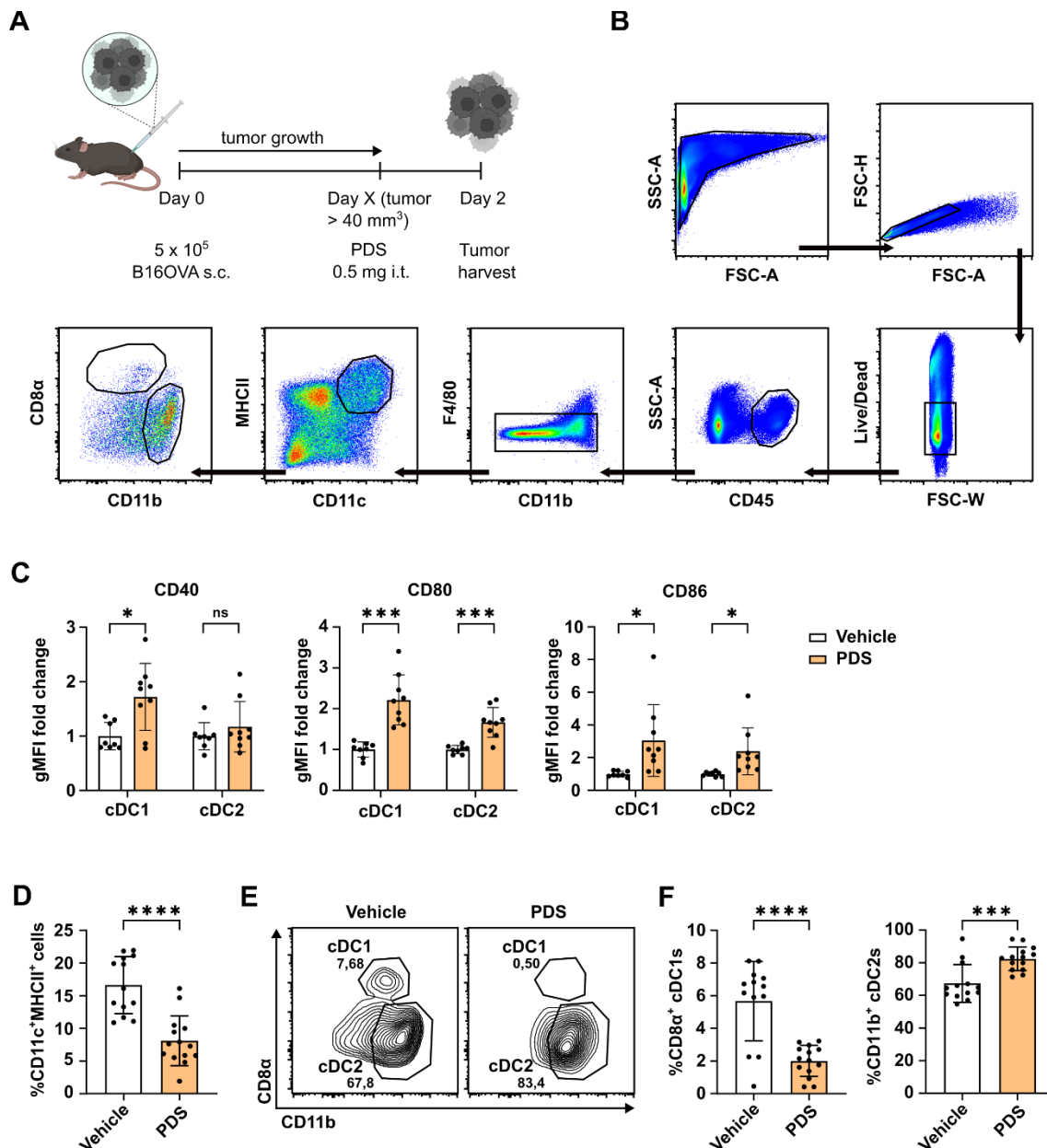


Figure 4.1 | Intratumoural injection of PDS induces DC activation in B16OVA melanoma. A) Experimental setup showing intratumoural pyridostatin (PDS) treatment in B16OVA-bearing mice. C57BL/6N wild-type mice were injected with B16OVA melanoma cells subcutaneously (s.c.). On day X (tumour size > 40 mm³), 0.5 mg PDS was injected intratumourally (i.t.). The tumour was harvested on day 2 after PDS injection. B) Representative gating strategy for dendritic cells (DCs) *in vivo*. Conventional type I dendritic cells (cDC1s) are characterised based on CD11c⁺MHCII⁺CD8⁺CD11b⁻ expression, while conventional type II DCs (cDC2s) are determined by CD11c⁺MHCII⁺CD8⁻CD11b⁺ expression. C) Surface expression levels of co-stimulatory molecules (CD40, CD80, CD86) on cDC1s and cDC2s in PBS (vehicle)- and PDS-injected tumours. Changes in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change normalised to vehicle. D) Percentage of CD11c⁺MHCII⁺ DCs in vehicle-

and PDS-injected tumours. E) Representative contour plots display the proportion of CD8⁺ cDC1s and CD11b⁺ cDC2s in vehicle- and PDS-injected tumours. F) Percentage of CD8⁺ cDC1s and CD11b⁺ cDC2s in vehicle- and PDS-injected tumours. Data are represented as mean $\pm$ SD based on $n = 3$ independent experiments. Significance was calculated using multiple student's t-tests corrected for multiple comparisons using the Holm-Šidák method (C) and single student's t-tests (D, F). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DC activation due to the recognition of tumour antigens is followed by their migration to tumour dLNs (tdLNs). Within dLNs, DCs present tumour-specific antigenic peptides to naïve T cells, thus inducing an anti-tumour immune response. Cytotoxic CD8⁺ T cells are considered the main T cell subset in the elimination of cancer cells. CTLs are primarily activated by cDC1s through cross-presentation of tumour antigens via MHC I (Hildner et al., 2008; Roberts et al., 2016).

Due to the decreased proportion of cDC1s in PDS-injected tumours, I assumed that these cells migrated towards tdLNs for T cell priming. Therefore, I assessed DC migration from PDS-injected tumours towards the dLNs by measuring G4 levels in DCs isolated from inguinal and iliac lymph nodes using the BG4 antibody. In the case of DC migration to LNs, I expected increased G4 levels in DCs isolated from PDS-injected tumour-bearing mice compared to those isolated from vehicle-injected tumour-bearing mice. G4 levels were significantly increased about 1.2-fold in DCs isolated from the dLNs of PDS-injected tumour-bearing mice (Figure 4.2 A, B), suggesting that following intratumoural injection of PDS, activated DCs might migrate towards the dLNs to induce anti-tumour immunity.

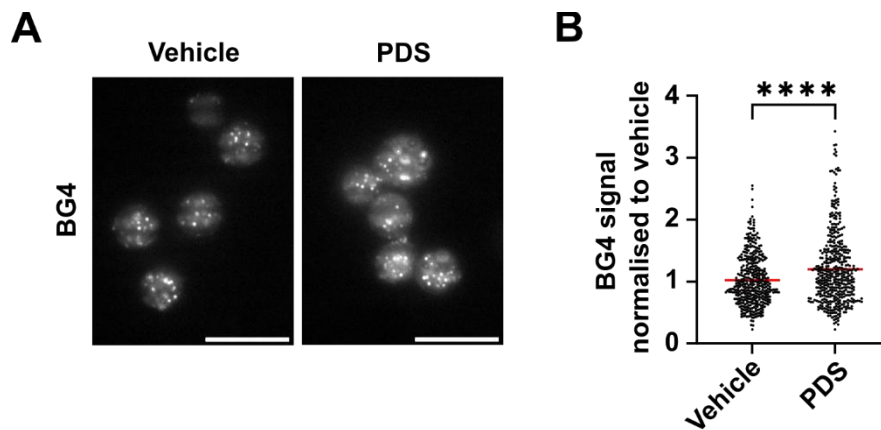


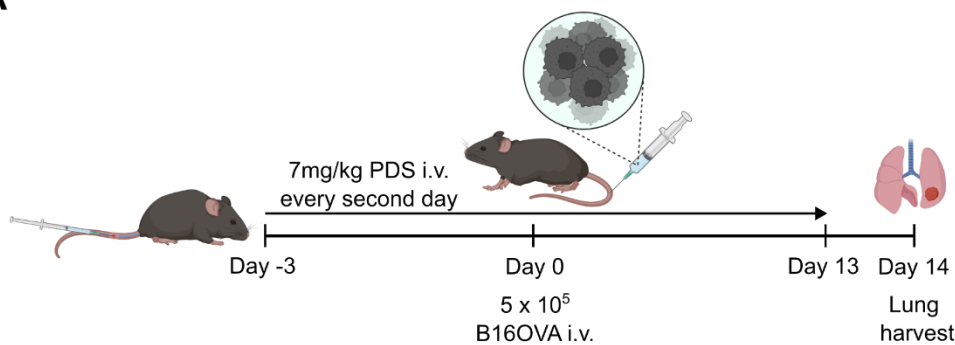
Figure 4.2 | G4 levels in DCs from tumour-draining LNs. A) Representative immunofluorescence (IF) microscopy image of G-quadruplex (G4) structures in dendritic cells (DCs) isolated from draining lymph nodes (LN) of vehicle- and pyridostatin (PDS)-injected tumour-bearing mice. G4 structures were labelled with the G4-specific single-chain antibody BG4 (white signal). Magnification 60 x, scale bar is 10 μ m. B) G4 levels determined by the fluorescent intensity of BG4 in nuclei normalised to vehicle. The red line represents the mean value. Results are based on three mice per group. Significance was calculated using student's t-test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.2.2 Intravenous injection of PDS induces DC activation in B16OVA lung metastasis

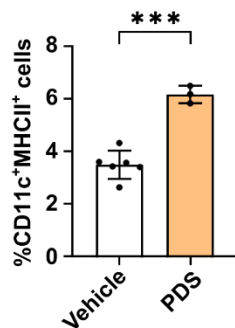
Since melanoma is known to metastasise, I also characterised the impact of G4 stabilisation by PDS on DCs in a cancerous environment of metastasis. Mice were injected with 5×10^5 B16OVA cells intravenously (i.v.) on day 0, to induce metastasis formation in the lung. PDS treatment was started three days prior to B16OVA cell injection (day -3). The concentration of 7 mg/kg PDS was chosen according to previous studies using PDS *in vivo* (Groelly et al., 2022). PDS was injected i.v. every second day. Lungs were harvested and DCs were characterised by flow cytometry on day 14 after tumour cell injection (Figure 4.3 A). In contrast to melanoma, in which a decrease in the total amount of DCs was observed, the proportion of total DCs was significantly increased (3.5% to 6.2%) in metastatic lungs of PDS-treated mice (Figure 4.3 B). Nevertheless, the proportional composition of the particular DC subsets revealed a significant reduction in the proportion of cDC1s (3.7% to 2.7%) linked to an increase in the proportion of cDC2s (30.6% to 42.4%) in metastatic lungs of PDS-injected mice

(Figure 4.3 C). Furthermore, DCs in metastatic lungs of PDS-treated mice displayed elevated expression levels of co-stimulatory molecules. CD40 was significantly enriched in cDC2s (1.7-fold), but also cDC1s showed positive regulation of CD40 (1.5-fold) following PDS treatment. While for CD80 only a positive trend was observed, CD86 was significantly induced in cDC1s (1.7-fold) and cDC2s (2-fold) in metastatic lungs of PDS-treated mice (Figure 4.3 D). These results indicate that G4 stabilisation by PDS does not only stimulate DC activation *in vitro* but also under inflammatory conditions *in vivo*.

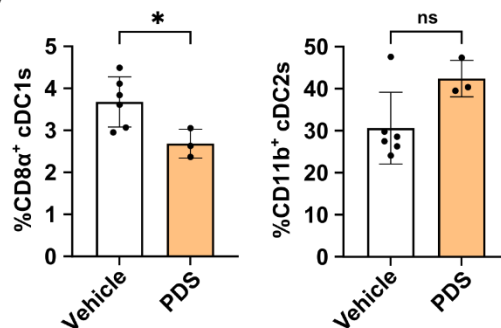
A



B



C



D

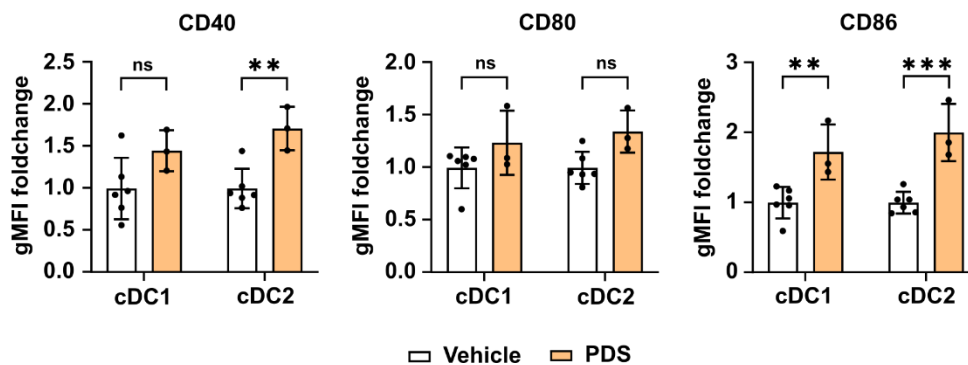


Figure 4.3 | Intravenous injection of PDS stimulates co-stimulatory molecule expression on DCs in B16OVA-bearing lungs. A) Experimental setup of B16OVA metastasis model. C57BL/6N wild-type mice were injected intravenously (i.v.) with B16OVA cells. Three days prior to B16OVA cell injection, treatment with 7 mg/kg pyridostatin (PDS) i.v. was started with additional injections every two days. Lungs were harvested on day 14 after B16OVA injection. B) Percentage of CD11c⁺MHCII⁺ DCs in metastatic lungs of PBS (vehicle)- and PDS-injected mice. C) Percentage of CD8⁺ cDC1s and CD11b⁺ cDC2s in metastatic lungs of vehicle- and PDS-injected mice. D) Surface expression levels of co-stimulatory molecules (CD40, CD80, CD86) on cDC1s and cDC2s in metastatic lungs of vehicle- and PDS-injected mice. Changes in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change normalised to vehicle. Normalisation to vehicle was performed by dividing the respective gMFI value by the gMFI mean of all vehicle mice. Data are represented as mean $\pm$ SD based on n = 1 independent experiment. Significance was calculated using student's t-tests (B, C) and multiple student's t-test corrected for multiple comparisons using the Holm-Šídák method (D). ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

4.2.3 Intraperitoneal injection of PDS does not affect mouse well-being in the early phase of GvHD development

Next, I assessed the impact of G4 stabilisation in aGvHD using an MHC major-mismatched aGvHD model, in which C57BL/6 (H-2Kb, female) served as donor and BALB/c (H-2Kd, female) as recipient. One day prior to allo-HCT (day -1), BALB/c mice were exposed to total body irradiation (TBI) using a dose of 2 x 2.5 gray (Gy), with a 4 h recovery phase in between. 6×10^5 CD3⁺ T cells and 5×10^6 CD90.2-depleted BM cells isolated from C57BL/6 mice were injected i.v. into BALB/c mice on day 0, leading to GvHD induction. PDS treatment was started two days prior to allo-HCT (day -2). 7 mg/kg PDS or H₂O (vehicle) were injected intraperitoneally (i.p.) every second day. Mice were monitored and scored for weight, skin, fur, activity and posture for up to 60 days (Figure 4.4).

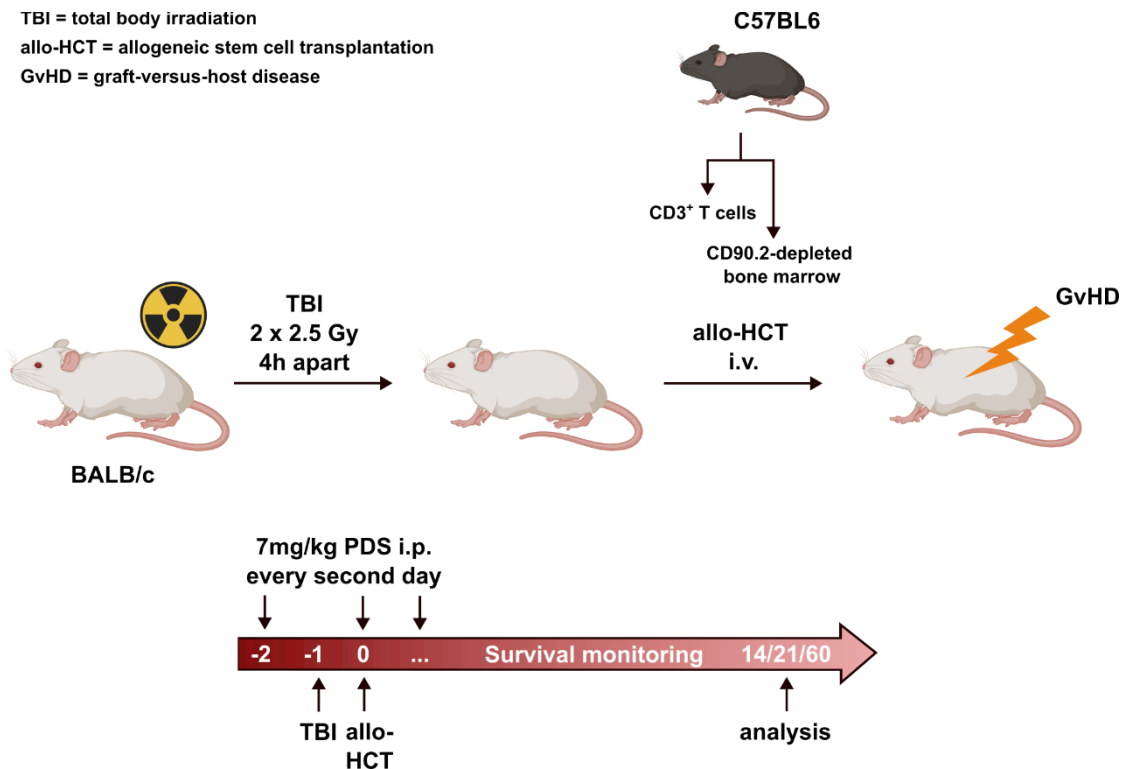


Figure 4.4 | Experimental setup of the GvHD model. BALB/c mice were exposed to total body irradiation (TBI) (2 x 2.5 Gy) on day -1. On day 0, BALB/c mice were transplanted with 90.2-depleted BM and CD3⁺ T cells from C57BL/6N via intravenous (i.v.) injection. Two days prior to transplantation (allo-HCT), pyridostatin (PDS) treatment was started (day -2). 7 mg/kg PDS was injected intraperitoneally (i.p.) every second day. Survival of mice was monitored for 14, 21 and 60 days following transplantation.

In order to characterise the impact of G4 stabilisation by PDS on the early onset of aGvHD, I monitored mice until day 21. Within the first week after allo-HCT, mice lost about 20 % of their original body weight, reaching the low on day 8 (Figure 4.5 A). Afterwards, mice regained up to 95 % of their original body weight, which was maintained until day 21. During this period, I did not observe any differences between the weight loss of vehicle- and PDS-injected mice. Further characteristics concerning mouse well-being, including fur, activity and posture progressively worsened in the first week after allo-HCT, indicated by an increasing overall clinical score (Figure 4.5 B). The overall score reached a peak on day 8 with a score sum of 5.5. Afterwards, the clinical score declined again and remained low until day 21, indicating ameliorated symptoms. Similar to the weight, the overall score did not reveal any differences between vehicle- and

PDS-treated mice. Together, these data indicate that G4 stabilisation by PDS does not affect the early progression of aGvHD until day 21.

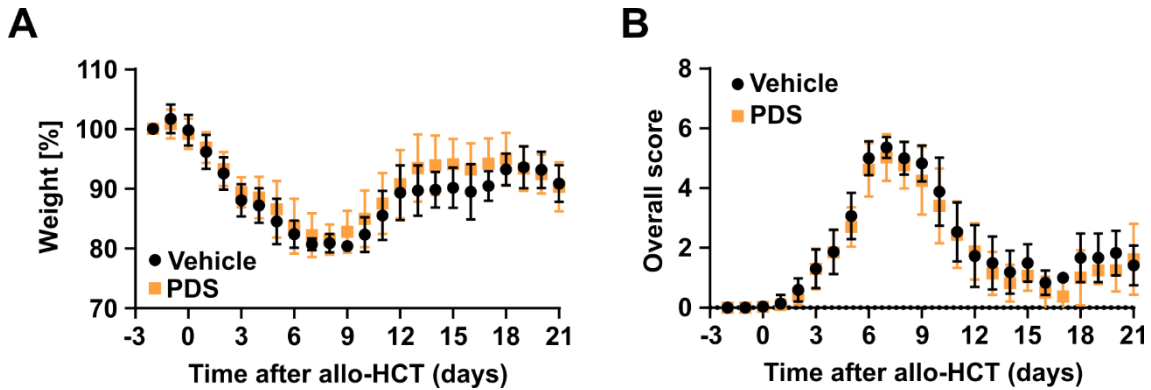


Figure 4.5 | Intraperitoneal injection of PDS does not affect mouse well-being during the early onset of aGvHD. BALB/c mice underwent total body irradiation (TBI) and transplantation (allo-HCT) for GvHD induction. Mice were injected with H₂O (vehicle) or 7 mg/kg pyridostatin (PDS) intraperitoneally (i.p.) every second day, starting two days before allo-HCT (day -2). Mice were monitored for up to 21 days after allo-HCT. A) Weight (%) of vehicle- and PDS-injected mice monitored for 21 days after allo-HCT normalised to the original body weight. B) Overall score including weight loss, fur, posture and activity of vehicle- and PDS-treated mice monitored for 21 days after allo-HCT. Data are represented as mean $\pm$ SD based on n = 2 independent experiments.

In addition to phenotypical changes, I also investigated changes in the blood cell counts due to PDS treatment on day 14 after allo-HCT. Here, I did not detect any significant changes in the number of blood and immune cells (Figure 4.6). For blood cells, no differences in the number of leukocytes were observed, but a slight decrease in red blood cells and a positive trend for the number of platelets in mice injected with PDS (Figure 4.6 A). Furthermore, the percentage of eosinophils was slightly increased (13.4 % to 20.11 %) in the blood of PDS-treated mice (Figure 4.6 B). In contrast, the percentage of neutrophils was reduced (52.58 % to 46.87 %). The percentage of lymphocytes, monocytes and basophils in the blood was not affected by PDS.

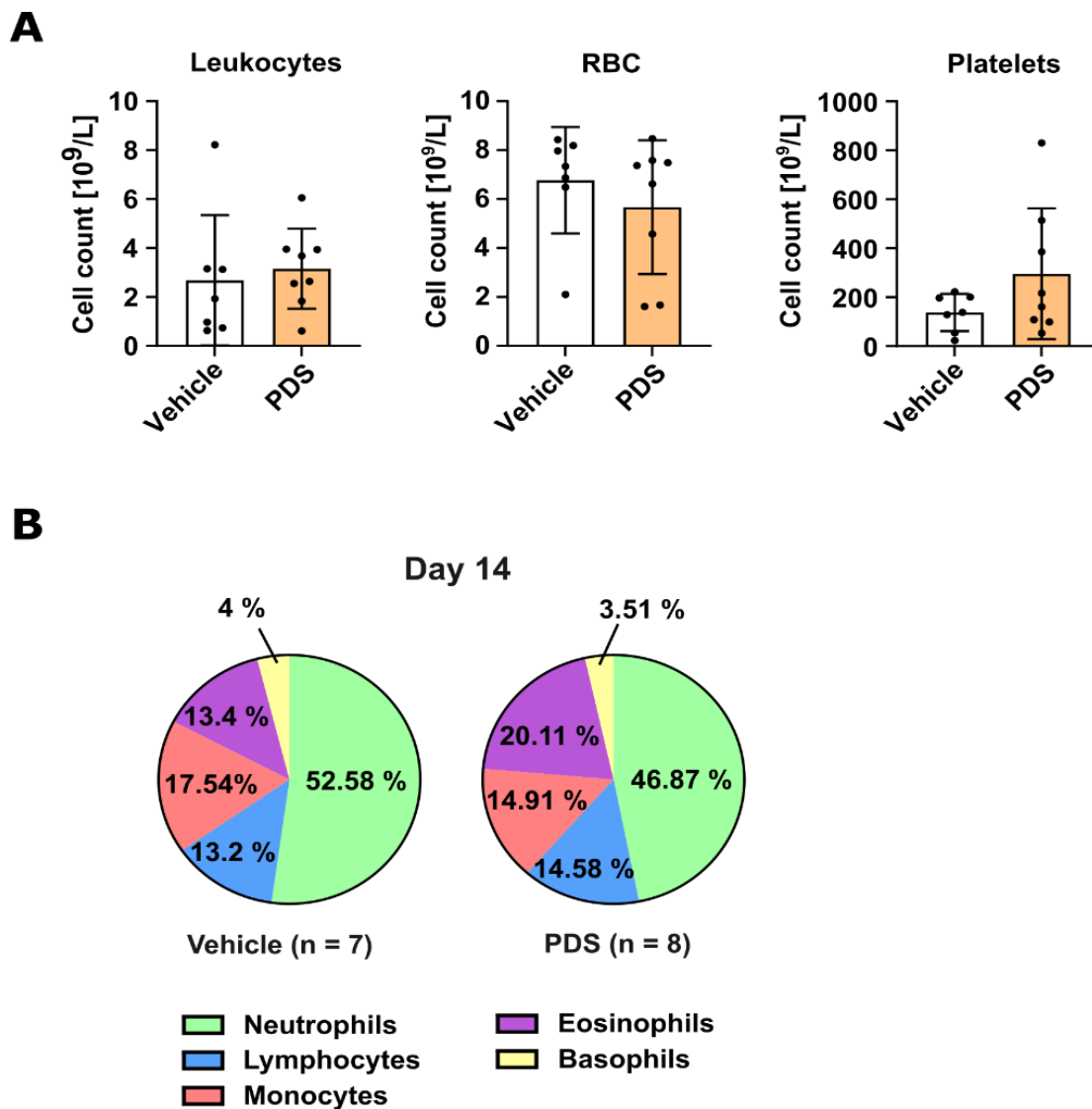


Figure 4.6 | Intraperitoneal injection of PDS does not affect the cellular composition of the blood during the early onset of aGvHD. BALB/c mice underwent total body irradiation (TBI) and transplantation (allo-HCT) for GvHD induction. Mice were injected with H₂O (vehicle) or 7 mg/kg pyridostatin (PDS) intraperitoneally (i.p.) every second day, starting two days before allo-HCT (day -2). On day 14 after allo-HCT, blood samples were collected from vehicle- and PDS-injected mice and analysed for the presence of blood and immune cells A) Number of leukocytes, red blood cells (RBC) and platelets in the blood of vehicle- and PDS-injected mice on day 14 after allo-HCT. Data are represented as mean $\pm$ SD based on n = 2 independent experiments. B) Percentages of neutrophils, lymphocytes, monocytes, eosinophils and basophils in the blood of vehicle- and PDS-injected mice on day 14 after allo-HCT. Data are represented as mean based on n = 2 independent experiments. Significance was calculated using student's t-tests. ns p > 0.05.

In order to assess the impact of G4 stabilisation by PDS on DCs in the early onset of GvHD, I characterised splenic DCs by flow cytometry on day 14 after allo-HCT. No difference in the proportion of DCs present in the spleen was detected between vehicle- and PDS-injected mice (Figure 4.7 A). Furthermore, also the proportional composition of DC subsets including CD8 α ⁺ cDC1s and CD11b⁺ cDC2s was not affected by PDS (Figure 4.7 B). A slight increase of about 4 % in B220⁺ pDCs was observed in PDS-treated GvHD-bearing mice (Figure 4.7 B). In addition, I assessed DC activation based on changes in the expression of the co-stimulatory molecules CD40, CD80 and CD86. CD40 expression was significantly elevated about 1.5-fold in cDC1s. In cDC2s, only a slight increase in CD40 surface expression was detected (Figure 4.7 C). Expression of CD80 was significantly decreased by about 50 % in both, cDC1s and cDC2s. For CD86, only a marginal trend for enhanced expression was observed (Figure 4.7 B). Together, these data indicate that PDS injection does not affect DC activation in the early onset of aGvHD.

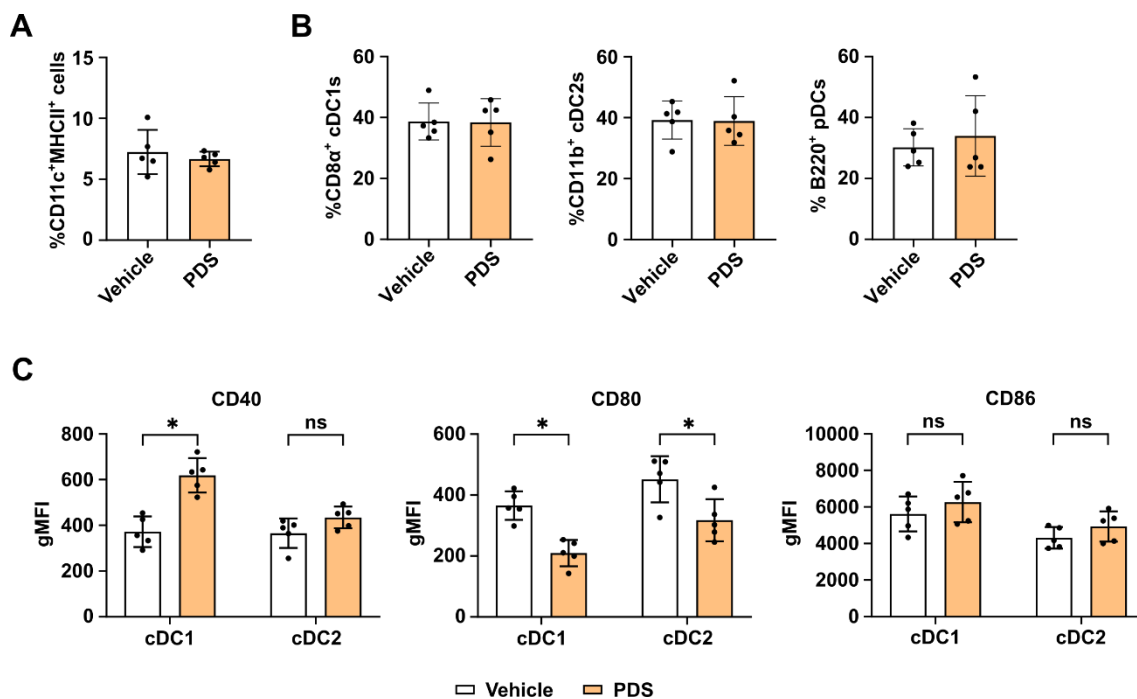


Figure 4.7 | Intraperitoneal injection of PDS does not affect splenic DCs in the early onset of aGvHD. BALB/c mice underwent total body irradiation (TBI) and transplantation (allo-HCT) for GvHD induction. Mice were injected with H₂O (vehicle) or 7 mg/kg pyridostatin (PDS) intraperitoneally (i.p.) every second day, starting two days before allo-HCT (day -2). On day 14 after allo-HCT, splenic

dendritic cells (DCs) of vehicle- and PDS-injected mice were characterised. A) Percentage of CD11c⁺MHCII⁺ DCs in spleens of vehicle- and PDS-injected mice. B) Percentage of CD8⁺ cDC1s, CD11b⁺ cDC2s and B220⁺ pDCs in spleens of vehicle- and PDS-injected mice. C) Surface expression levels of co-stimulatory molecules (CD40, CD80, CD86) on cDC1s and cDC2s in spleens of vehicle- and PDS-injected mice. Changes in surface expression are depicted as geometric mean fluorescent intensity (gMFI). Data are represented as mean $\pm$ SD based on n = 1 independent experiment. Significance was calculated using single student's t-tests (A, B) and multiple student's t-test corrected for multiple comparisons using the Holm-Šídák method (C). ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

4.2.4 Intraperitoneal injection of PDS reduces acute GvHD severity

Besides the impact of G4 stabilisation on the early onset of aGvHD, I was further interested in investigating, if PDS might impact the long-term progression of aGvHD. To characterise the impact of G4 stabilisation on aGvHD progression, I monitored survival and the occurrence of aGvHD-specific symptoms such as weight loss, (bloody) diarrhoea, skin rash and fur loss in GvHD-induced mice for 60 days.

PDS injection enhanced the survival of aGvHD-bearing mice, however, this effect was not significant (p-value = 0.087) (Figure 4.8 A). Nevertheless, up to day 30 after allo-HCT, 87.5 % of vehicle-treated GvHD-bearing mice were already dead, while 75 % of PDS-injected mice were still alive. 37.5 % of PDS-treated mice died between day 34 to day 41, indicating that PDS prolongs the survival of mice for at least one week compared to vehicle. By the end of the experiment (day 60 after allo-HCT), 37.5 % of PDS-injected mice were still alive, while only 12.5 % of vehicle-treated mice survived. This indicates that intraperitoneal injection of PDS enhances the survival of GvHD-bearing mice.

Similar to previous findings, mice lost about 20 % of their original body weight within the first week, reaching the low at day 8. Afterwards, mice regained up to 95 % of their initial weight until day 20. So far, no differences in the weight were observed between vehicle- and PDS-treated mice bearing GvHD. However, vehicle-treated mice started losing weight rapidly between day 20 to day 30 after allo-HCT (Figure 4.8 B). 7 out of 8 vehicle-treated mice lost > 20 % (stopping

criteria) of their original body weight until day 30. In comparison, only 2 out of 8 PDS-injected mice fell below the threshold of 80 % initial weight. The majority of PDS-injected mice maintained a stable body weight ≥ 80 % up to 60 days after allo-HCT (Figure 4.8 B).

In addition to weight loss, vehicle and PDS-treated GvHD-bearing mice revealed differences in the occurrence of skin rash and fur loss. Vehicle-treated mice showed an extensive loss of fur on the forehead, back and ventral side. In addition, vehicle-treated mice developed a scaly, chapped skin rash (Figure 4.8 C left). In contrast, PDS-injected mice did not extensively lose their fur (mainly a thinned-out region on the forehead was observed) and only developed slight scaly skin (Figure 4.8 C right).

The entirety of all monitored symptoms (e.g. weight loss, fur, skin, activity, posture) is summarised in the overall score. Similar to previous experiments, the overall score of both, vehicle- and PDS-injected mice, rapidly increased within the first week of GvHD induction up to a score sum of 5.5. Afterwards, the score declined again to 1, indicating a symptomatic enhancement. Following day 20 after allo-HCT, the overall score of vehicle-treated mice rapidly increased to 4.5 to 5.5 before day 30 in 7 out of 8 mice (Figure 4.8 D). This highlights the severe occurrence of aGvHD-specific symptoms in vehicle-treated mice. In contrast, PDS-injected mice revealed a constant score sum of about 2.5, which decreased over time (Figure 4.8 D). This underlines the lower occurrence of symptoms and minor severity of GvHD in PDS-injected mice.

Together, these data indicate that PDS reduces aGvHD severity by enhancing the survival and the occurrence of GvHD-specific symptoms including weight loss, fur loss and skin rash.

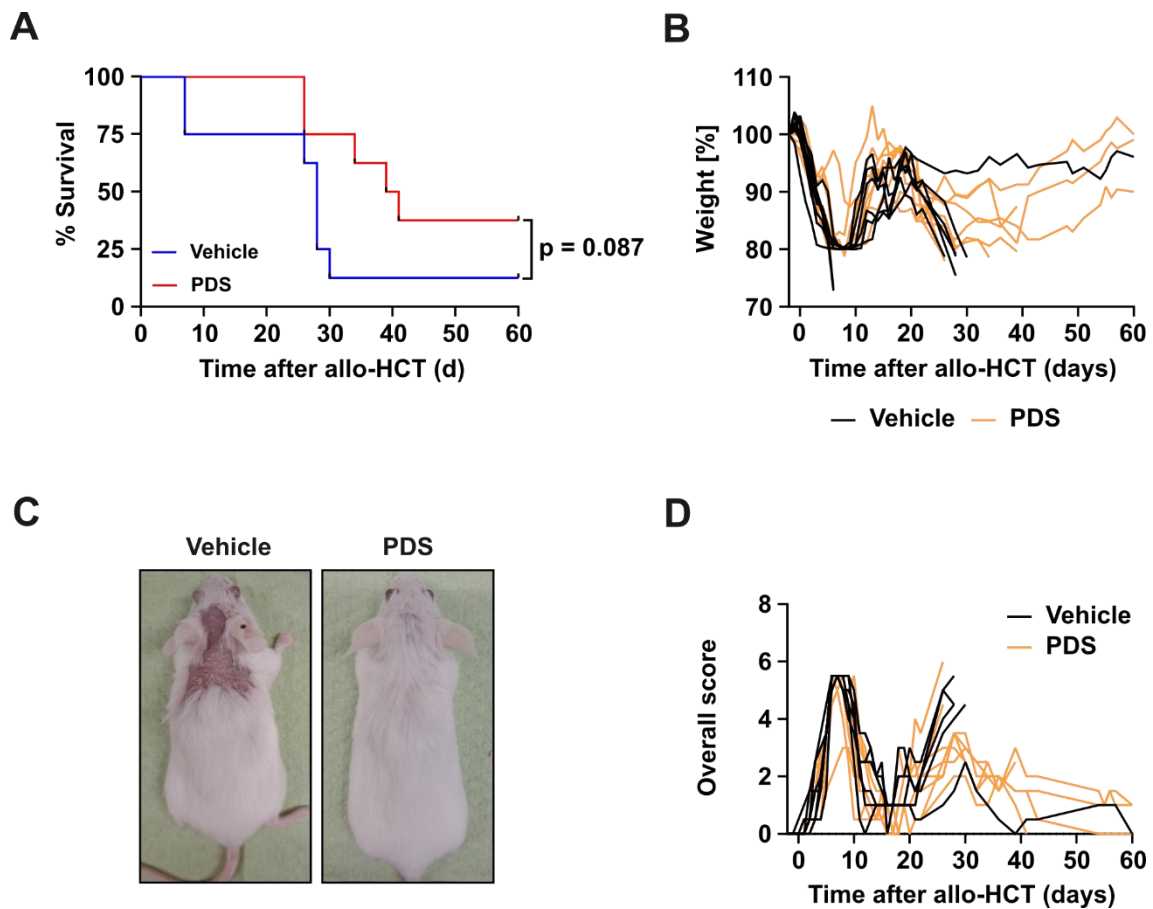


Figure 4.8 | Intraperitoneal injection of PDS improves survival and symptomatic progression of aGvHD. BALB/c mice underwent total body irradiation (TBI) and transplantation (allo-HCT) for GvHD induction. Mice were injected with H₂O (vehicle) or 7 mg/kg pyridostatin (PDS) intraperitoneally (i.p.) every second day, starting two days before allo-HCT (day -2). GvHD-bearing mice were monitored for 60 days to characterise the impact of PDS on the course of aGvHD. A) Survival rate of vehicle and PDS-treated GvHD-bearing mice. Significance was calculated based on simple survival analysis (Kaplan Meier) with the Mantel-Cox test. ns $p > 0.05$. B) Weight (%) of vehicle- and PDS-injected mice normalised to the original body weight. Each line represents an individual mouse. Ending lines indicate further exclusion from the experiment due to the achievement of stopping criteria C) Representative images of vehicle (left)- and PDS-injected mice (right) showing the severity of symptoms such as fur loss and skin rash. D) Overall score representing the entirety and severity of monitored symptoms (weight loss, fur, posture, activity and skin rash) in vehicle- and PDS-treated mice during aGvHD. Each line represents an individual mouse. Ending lines indicate further exclusion from the experiment due to the achievement of stopping criteria. Results are based on $n = 1$ independent experiment performed with 8 mice per group.

4.3 Discussion

DCs play a critical role in various clinical implications including anti-viral immunity, anti-tumour immunity and autoimmune diseases. Therefore, DCs have gained great attention as a potential target for therapeutic approaches. In this chapter, I investigated G4 stabilisation by PDS as an immunotherapeutic tool in the B16OVA melanoma and acute Graft-vs-Host-Disease mouse model. In both diseases, DCs play a critical role, however, the outcome of their function in tumour and GvHD is opposed. While DCs are important for the induction of an adequate anti-tumour immune response by cross-presenting tumour antigens via MHC I to CD8⁺ cytotoxic T cells (Roberts et al., 2016), in aGvHD, DCs are responsible for the induction of the disease by presenting self-antigens to alloreactive T-cells, resulting in host tissue damage (Duffner et al., 2004; Shlomchik et al., 1999).

4.3.1 Intratumoral injection of PDS in B16OVA melanoma induces DC activation

To characterise the impact of G4 stabilisation on tumour immunity, PDS (0.5 mg in 50 µl PDS) was injected intratumourally into B16OVA melanoma mice. This injection route was chosen based on previous studies conducted by Nils Kosiol (Heine Lab) showing that intravenous injection of PDS does not affect the tumour, while intratumoural injection of PDS reduces tumour growth and enhances response to checkpoint therapy (unpublished data).

Consistent with the *in vitro* data, intratumoural injection of PDS caused a significant upregulation of co-stimulatory molecules (CD40, CD80 and CD86) on cDC1s and cDC2s in B16OVA melanoma (Figure 4.1 C), demonstrating that PDS triggers DC activation within the tumour. These findings might be a consequence of PDS-mediated G4 stabilisation in DCs, as observed *in vitro*, and need to be further clarified by analysing G4 structure formation in tumour-associated DCs using BG flow (De Magis et al., 2021) or IF microscopy by staining G4 structures with the specific G4-binding antibody BG4 (Maurizio et al., 2024; Miglietta et al.,

2024). However, it needs to be considered that these findings are not only based on a direct effect of PDS-mediated G4 stabilisation in DCs but rather on a systemic effect caused by PDS cytotoxicity. Previous studies by Nils Kosiol revealed that only 5-10 % of tumour cells were alive *in vivo* 24 h after intratumoural injection of PDS (unpublished data). Induction of immunogenic cell death within the tumour leads to the release of TAAs which will be captured by DCs resulting in DC activation and maturation (Woo et al., 2014). Hence, DC activation within the tumour could be based on a direct effect of PDS through G4 stabilisation within DCs or an indirect effect due to PDS-induced cytotoxicity. The cDC1 population completely disappeared from the tumour after intratumoural injection of PDS (Figure 4.1 E, F). A possible explanation for the missing cDC1 population could be that due to intratumoural PDS injection, activated cDC1s migrated from the tumour to the tdLNs, where they cross-present TAAs to CD8⁺ T cells, to induce an anti-tumour immune response (Roberts et al., 2016). I hypothesised that DCs migrated from PDS-injected tumours reveal higher G4 levels compared to DCs isolated from dLNs of vehicle-injected tumours due to PDS-mediated G4 stabilisation. Indeed, DCs isolated from dLNs of PDS-injected tumours revealed higher G4 levels (Figure 4.2), indicating that DCs might have migrated from the tumour site to the dLNs. However, there is also a minor possibility that PDS is diffused from the tumour into the lymphatic system and was transported to the lymph nodes where it stabilised G4s in resident DCs. The use of fluorescently labelled PDS, such as Cy5-PDS (Ray et al., 2020) could identify DCs that have migrated from the tumour to the lymph nodes. Furthermore, the staining of G4 structures was performed in pan-DCs (pDCs, cDC1s, cDC2s) isolated from dLNs, thus the occurrence of G4 structures was not distinguished between cDC1, cDC2 and pDCs. Magnetic resonance imaging is one method to track the migration of DCs *in vivo* (Bulte and Shakeri-Zadeh, 2022). However, this method is based on *ex vivo* pre-labelling of DCs. Several studies described the use of fluorescently labelled antibodies for *in vivo* imaging and cell tracking (Krebs et al., 2018; Malide et al., 2014; Meyer et al., 2016; Paris-Robidas et al., 2011). Injection of fluorescently labelled XCR1-antibody into the tumour could enable a specific tracking of cDC1s and would allow the

identification of tumour-derived cDC1s in dLNs by IF microscopy. Besides microscopic imaging of DCs in dLNs, flow cytometric analysis could provide a better opportunity in regards to co-staining of several markers to distinguish different DC subsets and corresponding G4 levels. Microscopy is limited to only a few colours dependent on the laser composition and spectral overlay. With a spectrometer, several markers can be analysed at once and spectral overlay between the fluorophores can be compensated resulting in a clear segregation of the different colours. A protocol for co-staining of G4 structures with cell-specific markers has currently been established by our lab. Furthermore, blocking the migration of DCs from the TME by using either a CCR7 inhibitor or the exit blocking agent FTY720 could give further insights into DCs migration behaviour following intratumoural PDS injection (Han et al., 2015; Hos et al., 2016).

4.3.2 Does G4 stabilisation by PDS drive cDC1 reprogramming into cDC2-like cells?

Transcriptional changes in cDC1s due to deficiency of the main TF driving the cDC1 fate, have been shown to result in reprogramming of cDC1s into the cDC2 lineage (Lança et al., 2022). In addition, my *in vitro* data demonstrated that PDS induces transcriptional reprogramming in BMDCs (Figure 3.9). Besides the loss in cDC1s, the cDC2 population was increased in tumours injected with PDS (Figure 4.1 E, F). However, while several immune cells (NK cells, B cells, monocytes, T_{regs}, CD8⁺ T cells) were observed to infiltrate PDS-treated tumours, DCs did not (unpublished data by Nils Kosiol). Hence, apart from cDC1 migration from the tumour to tdLN, it is also possible that intratumoural injection of PDS drives cDC1 reprogramming into cDC2-like cells. Future work using single-cell analysis will shed light on the cDC2 background and the hypothesis of cDC1 reprogramming.

In conclusion, these data showed that intratumoural injection of PDS leads to DC activation within the tumour. Nevertheless, whether and how DC activation contributes to PDS-mediated tumour immunity needs to be further investigated.

4.3.3 The effect of PDS on eosinophils and neutrophils in aGvHD remains unknown

To characterise the impact of G4 structure formation on the course of aGvHD, mice were injected with 7 mg/kg PDS i.p. according to previous studies (Groelly et al., 2022), every second day. The intraperitoneal injection route was chosen over intravenous injection due to long-term administration for up to 60 days. PDS injection did not affect the early course progression of aGvHD (up to day 21) (Figure 4.5), showing that, in line with previous studies (Groelly et al., 2022), the concentration of PDS as well as the injection route does not harm the mice or cause side effects. Analysis of blood revealed a minor increase in eosinophils and a decrease in neutrophils (Figure 4.6 B). To date, the role of eosinophils in GvHD is not known. Nevertheless, the increase in blood eosinophils is a proposed biomarker for cGvHD and was first reported by Shulman in 1980 (Schultz et al., 2006; Shulman et al., 1980). Furthermore, increased numbers of blood eosinophils are also associated with aGvHD. Eosinophilia has been described to precede the development of aGvHD and is correlated with the severity of aGvHD after allo-HCT (Daneshpouy et al., 2002; McNeel et al., 2002; Paralkar et al., 2008; Shimomura et al., 2017). Intriguingly, several studies indicated that eosinophilia is associated with a less severe course of aGvHD and improved overall survival (Aisa et al., 2007; Imahashi et al., 2010). PDS induced the number of blood eosinophils, however, whether this is associated with a good or poor prognosis needs to be further investigated. Neutrophil infiltration into the intestinal tract following conditioning-induced tissue damage has been shown by multiple groups as one of the earliest events of aGvHD (Fischer et al., 2017; Schwab et al., 2014; Socié et al., 2004). Infiltration of neutrophils is correlated with a severe disease outcome due to neutrophil-mediated ROS production, exacerbating the tissue damage caused by the conditioning regimen (Schwab et al., 2014; Socié et al., 2004). Furthermore, neutrophils migrate from the intestine to mesenteric lymph nodes, where they initiate donor T cell expansion and GvHD development through antigen presentation (Hülsdünker et al., 2018). Hence, possible explanations for the decrease in blood neutrophils following PDS injection in the GvHD model include that neutrophils either die due to sensitivity towards PDS or

PDS might stimulate neutrophil infiltration into tissues such as the intestine. Experimental approaches to determine PDS-based neutrophil influx into the intestine involve flow cytometric analysis of neutrophil frequency or immunohistochemical staining of neutrophils in the gut.

4.3.4 Intraperitoneal injection of PDS does not affect splenic DCs during early onset of aGvHD

Based on my previous findings showing that PDS induces DC activation *in vitro* and within the tumour, I expected that injection of PDS would also result in DC activation in GvHD, which due to their role in GvHD initiation, would worsen the disease course. However, i.p. injection of PDS did not result in a distinct DC activation in the GvHD setting. Interestingly, the co-stimulatory molecule CD80 was significantly downregulated on splenic DCs (Figure 4.7 C). T_{regs} play a critical role in the prevention of GvHD through CTLA-4-dependent downregulation of CD80 on DCs (Bolton et al., 2015). A possible explanation for reduced CD80 expression on DCs in PDS-injected GvHD mice could be that similar to my previous observations in DCs, PDS induces T_{reg} development and activation, which then interact with DCs and prevent their maturation or ability of T cell priming through downregulation of co-stimulatory molecules. However, the impact of PDS on T_{regs} is unknown so far and needs to be further explored. In addition, it needs to be considered that the impact of PDS on DC activation in GvHD has been investigated only once and needs to be repeated to confirm the observed outcome. Nonetheless, to draw a conclusion on the impact of PDS injection on DCs in aGvHD the following points need to be considered: The performed experiment focused on the impact of PDS on splenic DCs. So far, it is not known if PDS can diffuse from the peritoneal cavity to the spleen or whether PDS is metabolised. Hence, it remains unclear if the previous observations are due to a direct effect of PDS. The use of fluorescently labelled PDS (e.g. Cy5-PDS) (Ray et al., 2020) would enable its tracking within the mice and could provide insights into whether PDS is taken up by splenic DCs or not. DCs take up self-antigens from the damaged tissues e.g. colon and migrate to the

mesenteric lymph nodes to prime donor T cells (Koyama et al., 2015). Therefore, investigating the effect of PDS on DCs in GvHD target tissues (skin, liver, gut) rather than the spleen would be of great interest to determine the effect of PDS on GvHD initiation. Moreover, factors such as time point of analysis, PDS concentration and PDS half-life need to be considered. Here, DCs have been characterised on day 14 following allo-HCT. Analysis of DCs at multiple time points (e.g. day 7 and day 21) enhances the probability of monitoring DC activation. PDS has already been successfully used *in vivo* at a concentration of 7.5 mg/kg/day (i.v.) without causing toxicity (Groelly et al., 2022). In line with this, no adverse effects were observed by i.p. injection of PDS. However, it has not been addressed so far, whether the concentration of PDS used is efficient in stabilising G4s *in vivo*. In addition, the half-life of PDS is not known yet. Therefore, analysis of G4 structure stabilisation could provide insights into 1) whether the PDS concentration is sufficient to stabilise G4 structures *in vivo* and 2) if PDS administration in a two-day interval is enough to retain the effect.

4.3.5 Intraperitoneal injection of PDS reduces the severity of aGvHD

Long-term administration of PDS improved the survival and symptomatic progression of aGvHD in mice (Figure 4.8). However, the mechanism underlying PDS-mediated suppression of aGvHD remains to be elucidated. Due to its cell permeability, PDS can potentially affect a wide range of cell types. Nevertheless, the effect of PDS on distinct cells due to G4 stabilisation is mainly unknown. Among others, pDCs have been reported to play a crucial role in preventing severe GvHD (Hadeiba et al., 2008; Hassan et al., 2019; Lu et al., 2012). CCR9⁺ pDCs and pre-pDCs can promote T_{reg} expansion and suppress antigen-specific immune responses, thereby exerting a tolerogenic effect on donor T cell activation against host tissues (Lu et al., 2012). Transcriptome-wide analysis of BMDCs demonstrated induced expression of various anti-viral genes. Given the central role of pDCs in anti-viral immunity (Cervantes-Barragan et al., 2012), these data suggest that PDS may activate pDCs *in vitro*. However, this needs to be further investigated by flow cytometric analysis. Nevertheless, G4-mediated

stimulation of pDCs *in vivo*, resulting in a tolerogenic environment, could offer a plausible explanation for the improved course of aGvHD in PDS-treated mice. Preclinical studies revealed that allo-HCT is associated with a deficit in all DC subsets, which correlates with the occurrence of severe GvHD (Banovic et al., 2009; Reddy et al., 2004; Wikstrom et al., 2015). Shono et al. (2010) demonstrated that GvHD impairs the reconstitution of DCs, as GvHD-associated inflammatory responses damage the BM niche, which leads to dramatically decreased haematopoiesis including reduction of CDP. Thus, promoting DC reconstitution after allo-HCT could represent a promising strategy to re-establish protective immunity. Recently, Zyner et al. (2022) reported that G4s in human embryonic stem cells are linked to cellular differentiation based on transcriptional stabilisation and epigenetic modulation. Accordingly, PDS-mediated G4 stabilisation linked to stem cell differentiation and DC reconstitution, especially of pDCs, offers an alternative explanation for the reduced severity of aGvHD. In this context, single-cell analysis could provide further insights into the cellular changes associated with PDS treatment. Although long-term administration of PDS demonstrated to improve the survival and symptomatic progression of aGvHD in mice, it is important to note that these results are based on a single independent experiment and need to be repeated to validate the observed outcomes. Nevertheless, these data suggest that while PDS does not prevent aGvHD, it can reduce its severity.

Due to the immunostimulatory effect of PDS observed in tumours, this finding was rather unexpected and requires further investigation to understand how the opposite effects of promoting tumour rejection on one hand and suppressing GvHD on the other can result from one treatment. Possible explanations may include differences in the route of administration (intratumourally vs. intraperitoneal) as well as the influence of the local environment. In the B16OVA melanoma model, PDS was administered directly into the tumour. The cytotoxic effect of PDS led to tumour cell death, resulting in the release of tumour antigens, activation of immune cells, such as DCs and T cells and the induction of an anti-tumour immune response (unpublished data by Nils Kosiol). In contrast, in the GvHD model, PDS was administered intraperitoneally. Due to its permeability,

PDS can affect multiple cell types in various tissues. As each cell type carries out distinct functions, it is essential to investigate the systemic effects of PDS on specific immune cell populations to fully understand the mechanism underlying PDS-mediated attenuation of GvHD progression. As mentioned previously, pDCs and T_{reg} cells have been reported to exert a tolerogenic effect on donor T cells attacking host tissues (Lu et al., 2012). A possible explanation for the observed GvHD suppression is that systemic PDS administration may affect these regulatory cells in the blood, lymphoid organs or peripheral tissues, promoting their activation and migration to inflammatory sites (target organs). There, they could create a more tolerogenic environment, resulting in improved clinical outcomes in aGvHD. To further clarify the mechanism underlying PDS-mediated suppression of aGvHD, future experiments should explore the effect of PDS on different types of immune cells involved in both, induction and regulation of GvHD, including neutrophils and T cells, T_{regs} and pDCs, in both target and non-target organs.

5. Results – The role of G-quadruplexes in the regulation of PD-L1 expression in dendritic cells

5.1 Introduction

The PD-1/PD-L1 immune checkpoint represents a negative regulator of the immune system by inhibiting T cell immune function (Freeman et al., 2000). PD-L1 expressed on tumour cells and various immune cells, including APCs such as macrophages and dendritic cells, interacts with its receptor PD-1 mainly expressed on T cells, resulting in inhibition of T cell activation. Under steady-state conditions, PD-L1 is critical for the regulation of peripheral tolerance and autoimmunity by inhibiting self-reactive T cells and promoting the development of T_{regs} (Francisco et al., 2009; Keir et al., 2006; Probst et al., 2005). However, in inflammatory conditions, like in the tumour microenvironment, the PD-1/PD-L1 pathway plays an important role in cancer immune evasion by inhibiting T cell-mediated anti-tumour immune responses (Blank et al., 2004; Dong et al., 2002; Taube et al., 2012). Therefore, the PD-1/PD-L1 pathway has gained great attention, as a potential target for anti-cancer immunotherapies (Hirano et al., 2005). Most mechanisms of actions of PD-1/PD-L1 targeting agents focus on the effect on PD-1 expressing T cell activity. However, PD-L1 signalling in tumour cells was observed to promote tumour cell survival by inhibiting IFN-induced toxicity (Gato-Cañás et al., 2017). Therefore, it is of important need to additionally understand the processes that regulate PD-L1 expression and signalling to improve current therapeutic approaches.

Interestingly, the clinical response to anti-PD-L1 blockade was strongest when PD-L1 was expressed by tumour-infiltrating immune cells rather than by tumour cells (Herbst et al., 2014). A current study by Peng et al. (2020) described that tumour-infiltrating DCs express high levels of the inhibitory molecule PD-L1, switching from an immunostimulatory to an immunosuppressive state by limiting anti-tumour immune responses. In this study, they observed that the therapeutic effect of PD-L1 blockade was gone in a DC-conditional knockout mouse, although other cells still expressed high levels of PD-L1. This indicates a key role for PD-

L1 on DCs in response to PD-L1 blockade therapy. Besides PD-1, PD-L1 on DCs was also shown to interact with CD80 *in cis*, thus preventing it from binding to PD-1 to inhibit T cell activation (Sugiura et al., 2019; Y. Zhao et al., 2019; Zhao et al., 2018). However, PD-L1 is expressed much more abundantly on tumour-associated DCs than CD80, suggesting that PD-L1 on DCs still works to inhibit T cell activation via interaction with PD-1. Nevertheless, Mayoux et al. (2020) reported that blocking of PD-L1 on DCs interrupted CD80 sequestration *in cis*, which allowed CD80/CD28 interaction to enhance T cell priming. Based on the important role of PD-L1 on DCs in regulating anti-tumour immune responses, targeting PD-L1 regulation on DCs could provide a potential therapeutic strategy in anti-cancer immunity.

PD-L1 is mainly regulated via the PI3K/AKT and mitogen-activated protein kinase (MAPK) signalling pathways and can be controlled by several intracellular and extracellular signals (Atefi et al., 2014; Jiang et al., 2013; Liu et al., 2007; Song et al., 2013). IFN γ has been considered one of the most prominent inducers of PD-L1 expression (Abiko et al., 2015; Chen et al., 2019). Additionally, PD-L1 expression can be stimulated by various cytokines (TGF- β , TNF- α), interleukins (IL-1 β , IL-2, -4, IL-6, IL-10, IL-15; IL-17, IL27) and TLR ligands (LPS, Poly I:C) (Sun et al., 2018b). These external stimuli induce specific signalling cascades resulting in the recruitment of various TFs to the PD-L1 promoter, including Myc, HIF1 α , NF κ B, STAT1, IRF1 and STAT3, required to induce PD-L1 expression (Bellucci et al., 2015; Casey et al., 2016; Gowrishankar et al., 2015; Lee et al., 2006; Marzec et al., 2008; Noman et al., 2014).

G4 structures are highly overrepresented in gene promoter regions. About 50 % of human genes, especially oncogenes, contain G4 motifs close to their promoter regions, which have been reported to affect gene expression (Cogoi and Xodo, 2006; Huppert and Balasubramanian, 2007; Siddiqui-Jain et al., 2002; Chen et al., 2024; Li et al., 2023; Shen et al., 2021). G4 structures can have a positive and negative effect on gene transcription depending on which strand they are forming in. If the motif is encoded on the template strand, the G4 structure can inhibit transcription by blocking the transcription machinery, while G4 formation in

the non-template strand can enhance transcription by keeping the transcribed strand in a single-stranded conformation (Agarwal et al., 2014). Moreover, proteins binding to G4 structures can also affect gene transcription, such as transcriptional activators/repressors (Qin and Hurley, 2008). Computational studies revealed that predicted TF binding sites are proximal to or overlap with G4 motifs in gene promoters (Eddy and Maizels, 2008; Hou et al., 2019; Spiegel et al., 2021; Todd and Neidle, 2008). Amongst others, one TF whose binding site was reported to associate with G4s at promoters is IRF1, a TF involved in PD-L1 regulation (Spiegel et al., 2021). Genome-wide mapping of endogenous G-quadruplex DNA structures in the mouse genome identified a PQS in the promoter region of the PD-L1 gene (Marsico et al., 2019). Due to the potential role of G-quadruplexes in gene expression, I assumed that G4 stabilisation in the PD-L1 promoter may modulate PD-L1 expression and thus its function on inhibiting T cell activation. Therefore, G4 stabilisation represents a promising tool to regulate PD-L1 expression on the genomic level.

In this chapter, I aimed to unveil the mechanism of how G4 formation modulates changes in DCs by the example of PD-L1. First, I assessed the formation of the putative G4 motif identified within the PD-L1 gene promoter *in vitro* and *in cellulo* by Thioflavin T (ThT) staining, circular dichroism (CD) and ChIP using the G4 specific antibody BG4. In addition, I determined the impact of G4 stabilisation on PD-L1 regulation by evaluating changes in the PD-L1 gene, protein and surface expression level. Finally, I investigated proteins that interact with the G4 in the PD-L1 promoter by pulldown or electrophoretic mobility shift assay (EMSA).

Getting a better understanding of the mechanistic background of G4-mediated PD-L1 regulation might help to improve current immunotherapeutic strategies by providing a promising target on the genomic level.

5.2 Results

5.2.1 The putative G-quadruplex sequence in the PD-L1 promoter adopts a G4 structure *in vitro* and *in cellulo*

Marsico et al. (2019) published whole-genome maps of DNA G4 structures in multiple species, generated using ChIP and NGS. Genome-wide mapping of DNA G4 structures in the mouse genome identified multiple PQS in the PD-L1 gene under G4 stabilising conditions (Figure 5.1). Most of these potential G4 motifs are located in introns. Interestingly, one of the putative G4 sequences is located in the promoter region of the PD-L1 gene (Figure 5.1).

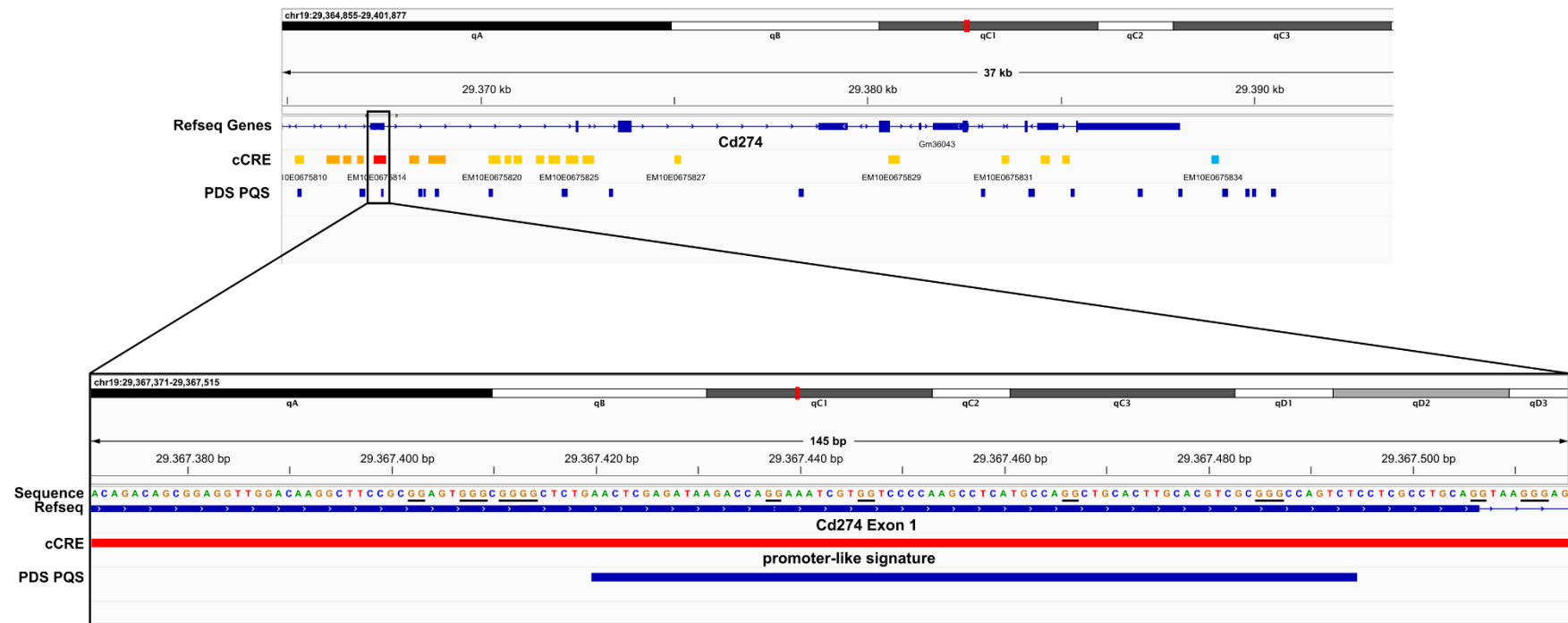


Figure 5.1 | A PQS located in the gene promoter of PD-L1. Whole genome mapping of DNA G4 structures by Marsico et al. (2019) identified a PQS (blue) in the promoter region (red) of the PD-L1 gene. cCRE = candidate cis-regulatory element.

To characterise, if the G4 motif identified in the gene promoter of PD-L1 can adopt a G4 structure *in vitro*, I performed a ThT staining using oligonucleotides composed of the PD-L1 promoter G4 motif sequence and scrambled control (Table 2). Thioflavin T is a benzothiazole previously used to detect amyloid fibrils (Rogers, 1965; Saeed and Fine, 1967). Renaud De La Faverie et al. (2014) developed a method using ThT to rapidly determine whether an oligonucleotide adopts a G4 conformation. They reported that ThT can be used as a dye to visualise G4 structures in a non-denaturing acrylamide gel, as the fluorescence intensity of ThT increases when it is bound to a G4 structure.

DNA oligonucleotides were incubated in G4 folding buffer (10 mM Tris pH 7.5, 100 mM KCl), to adopt a G4 confirmation. As a control for G4 formation, a G4-forming and non-G4-forming oligonucleotide were used. G4 formation was determined on a ThT-stained non-denaturing acrylamide gel by detecting ThT fluorescent intensity. The G4-forming oligonucleotide (positive control) revealed a strong fluorescent signal, while only a weak signal was observed for the non-G4-forming oligonucleotide (negative control), confirming the use of ThT for the detection of G4 structures (Figure 5.2 A left). Furthermore, a fluorescent signal was observed for the PD-L1 G4 oligonucleotide, demonstrating that the G4 motif in the PD-L1 promoter adopts a G4 confirmation *in vitro*. In contrast, a weak signal was detected for the scrambled control. Since the scrambled oligonucleotide sequence does not contain any G- or C-tracts required for G4 structure formation, the weak signal observed points towards an artefact. To further validate that the fluorescent signal detected with ThT derived from the DNA oligonucleotides, the acrylamide gel was additionally stained with ethidium bromide. Here, the position of the detected DNA molecules overlapped with the fluorescent signal observed following ThT staining (Figure 5.2 right). This confirms that the fluorescent signal originates from the DNA oligonucleotides.

Formation of the PD-L1 promoter G4 *in vitro* was additionally validated by CD spectroscopy. CD is a primary tool used for the characterisation of G4 structures. Various G4 structures arising from differences in G-quartet stacking, strand orientation and loop arrangements display individual CD spectra. General CD

spectral features have been accepted for particular G4 topologies to characterise and validate G4 formation: parallel (max: ~ 260 nm, min: ~ 240 nm), anti-parallel (max: ~ 290 nm, min: ~ 260 nm), or hybrid (max: ~ 290 nm, max: ~ 260 nm, min: ~ 240 nm) (Masiero et al. 2010; Paramasivan et al. 2007; Randazzo et al. 2013; Vorlíčková et al. 2012).

I validated the G4 formation of the identified PQS in the PD-L1 gene promoter by CD using oligonucleotides composed of the PD-L1 promoter G4 motif sequence, a mutated PD-L1 G4 and the scrambled control (Table 2). Prior to CD spectroscopy, oligonucleotides were incubated in G4 folding buffer to facilitate G4 structure formation. The CD spectra obtained from the PD-L1 promoter G4 (black) revealed a low peak at ~ 243 nm and a high peak at ~ 277 nm (Figure 5.2 B). These spectra resemble the CD spectral features of a parallel G4, indicating that the PD-L1 promoter G4 adopts a parallel G4 formation. In comparison, the CD spectra from the scrambled (blue) and mutated (red) G4 Oligos shifted to the same extent, displaying a low peak at ~ 250 nm and a high peak at ~ 280 nm. The scrambled control does not contain any G-runs and is therefore not able to adopt a G4 structure. Since scrambled control and mutated G4 displayed the same CD spectral features, it can be suggested that both sequences do not adopt a G4 structure but might fold into other secondary structures. Altogether, ThT staining and CD spectroscopy demonstrate that the potential G4 motif in the PD-L1 promoter region adopts a G4 structure *in vitro*.

To verify, if the G4 motif in the PD-L1 promoter region also forms in chromatin, I performed ChIP in MuTu DC1940 cells as described by Hänsel-Hertsch et al. (2018) using the G4-specific single-chain antibody BG4 to pull down DNA G4s. The MuTu DC1940 cell line was used due to the high amount of cells needed to prepare concentrated chromatin for the experimental procedure. Enrichment of PD-L1 promoter G4 DNA in untreated and MuTu cells treated with 0.5 μ M PDS was assessed by qPCR. Recovered PD-L1 promoter G4 DNA was significantly enriched at about 17 % in untreated MuTu DCs and 8 % in PDS-treated MuTu DCs compared to the BG4 negative control (no BG4) (Figure 5.2 C). These data

demonstrate that, besides *in vitro*, the G4 motif identified in the PD-L1 gene promoter is also forming *in cellulo*.

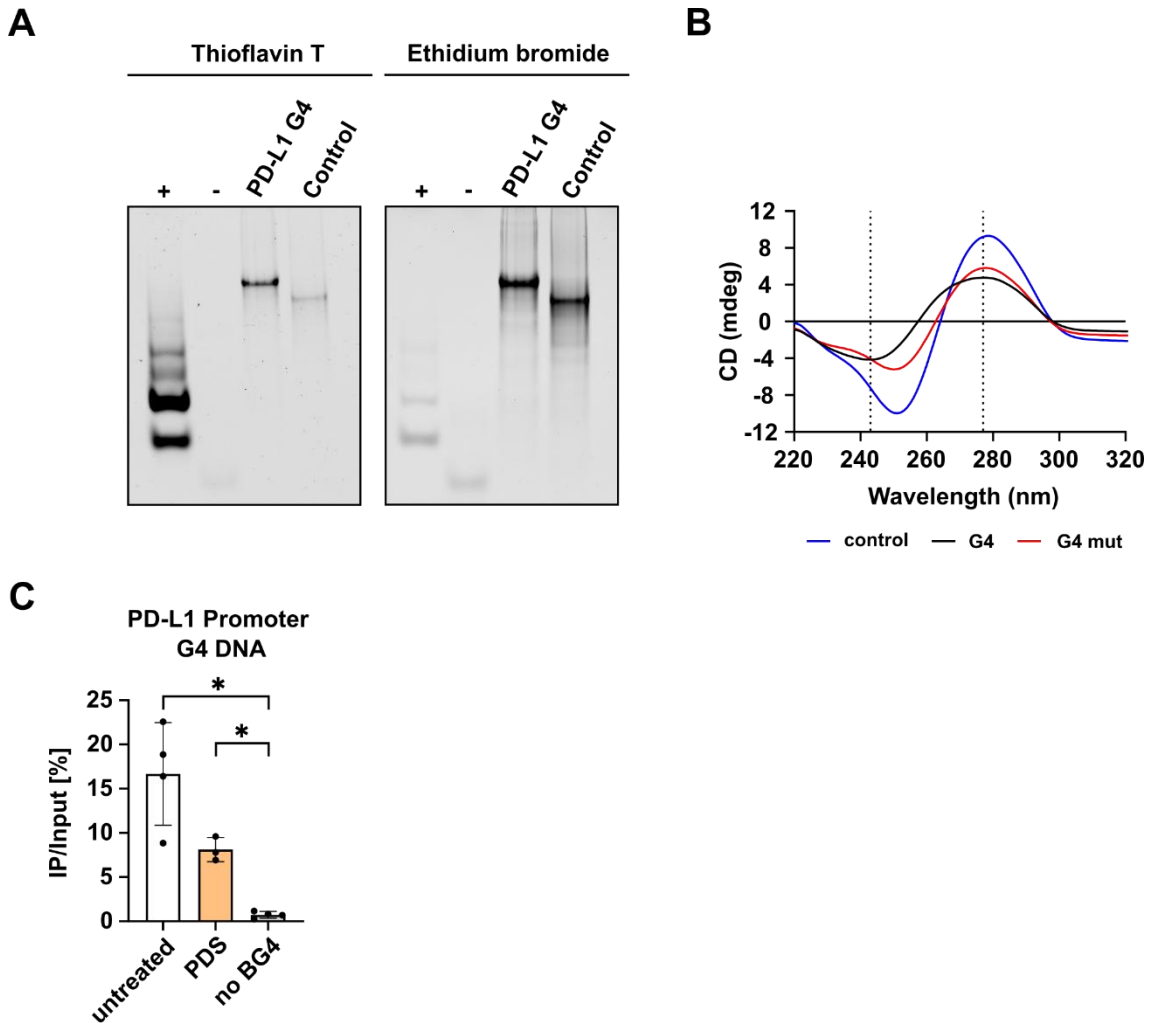


Figure 5.2 | The PD-L1 promoter G4 motif adopts a G4 structure *in vitro* and *in cellulo*. A) Thioflavin T (ThT) staining of the PD-L1 promoter G4 motif. Respective oligonucleotides were formed in G4 folding buffer (10 mM Tris pH 7.5, 100 mM KCl), separated by native gel electrophoresis and stained with 0.5 μ M ThT and 0.5 μ g/mL ethidium bromide. G4 structure formation was verified by the fluorescent signal intensity of ThT. B) CD spectra of PD-L1 promoter G4 (black), mutated G4 (red) and scrambled control (blue). Prior to CD spectroscopy, respective oligonucleotides were formed in G4 folding buffer (10 mM Tris pH 7.5, 100 mM KCl). C) G4 ChIP-qPCR for PD-L1 promoter G4 DNA in MuTu DC1940 cells. MuTu DCs were treated with 0.5 μ M PDS for 24 h. ChIP was performed according to Hänsel-Hertsch et al. (2018) using BG4. Enrichment of immunoprecipitated PD-L1 G4 DNA was assessed by qPCR. IP values were normalised to input. Data are represented as mean $\pm$ SD of at least $n = 3$ biologically independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test (C). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.2.2 The PD-L1 promoter G4 motif positively affects gene expression

G4 structure formation in gene promoter regions can either stimulate or inhibit gene transcription (Chen et al., 2024; Cogoi and Xodo, 2006; Du et al., 2008; Esain-Garcia et al., 2024; Hänsel-Hertsch et al., 2016; Siddiqui-Jain et al., 2002). To investigate, if the G4 motif in the PD-L1 promoter affects gene expression, I performed a Luciferase assay with the reporter plasmid Psi-CHECK2 (Promega), which contains a bicistronic construct of the Renilla and Firefly luciferase. The PD-L1 promoter G4 motif sequence, a mutated G4 sequence and the scrambled control were inserted downstream of the HSV-TK promoter in front of the Firefly luciferase via restriction-free cloning (Figure 5.3 A). The reporter constructs were transfected into HeLa cells since transfection greatly affects the viability of MuTu DCs (Fuentes Marraco et al., 2012). 1×10^4 HeLa cells were transfected with the reporter plasmids for 24 h. The ratio of Firefly activity to Renilla was determined according to the DualGlo Luciferase Assay protocol (Promega).

Luciferase activity was about 5-fold increased in the presence of the G4 motif compared to the scrambled control (Figure 5.3 B). In addition, the PD-L1 promoter G4 motif also resulted in an enhanced luciferase activity (2.4-fold) in contrast to the mutated G4 sequence. Together, these data indicate that the G4 motif in the PD-L1 promoter region has a positive effect on gene expression.

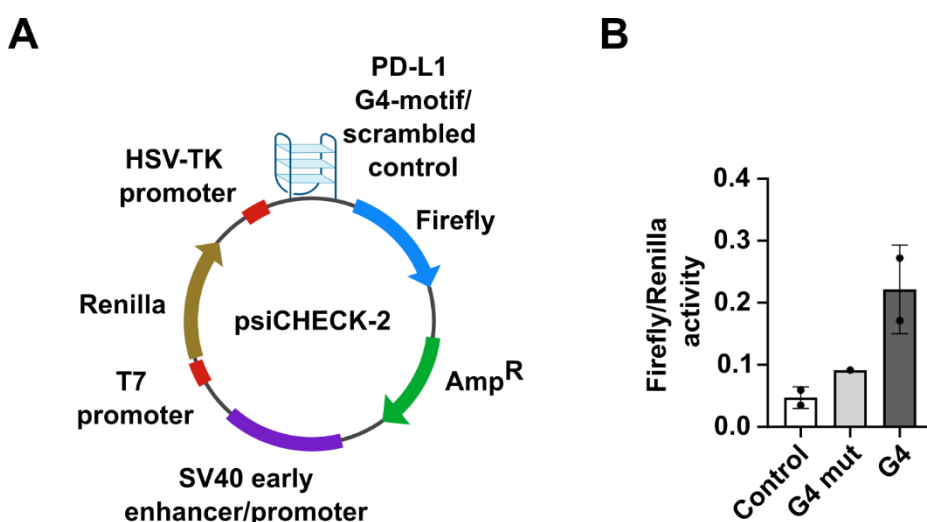


Figure 5.3 | The PD-L1 promoter G4 positively induces luciferase activity. A) PsiCHECK-2 plasmid map showing the position of the PD-L1 promoter G4 sequence, mutated G4 sequence and scrambled control inserted downstream of

the HSV-TK promoter in front of the Firefly luciferase. B) The ratio of Firefly activity to Renilla activity was measured using the DualGlo Luciferase Assay (Promega). Data are represented as mean $\pm$ SD of at least $n = 1$ biologically independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. ns $p > 0.05$

5.2.3 PDS induces PD-L1 expression in dendritic cells

Based on the previous findings showing that the G4 motif in the PD-L1 gene promoter can adopt a G4 structure *in vitro* and *in cellulo* and positively affects luciferase activity, I next investigated the effect of G4 formation on PD-L1 expression in BMDCs. BMDCs were generated as previously described (Figure 3.1 A). On day 7 of differentiation, immature BMDCs were treated with 10 μ M PDS for 24 h to induce G4 stabilisation.

The impact of G4 stabilisation on PD-L1 gene expression was assessed by 3' mRNA NGS. RNA sequencing analysis revealed a significant increase in the number of PD-L1 transcripts in PDS-treated BMDCs (Figure 5.4 A), showing that G4 stabilisation by PDS induces PD-L1 gene expression. Furthermore, the global protein level of PD-L1 was observed to be 4-fold increased in cDC1s and 4.4-fold upregulated in cDC2s following PDS treatment (Figure 5.4 B, C). The impact of G4 stabilisation on changes in PD-L1 surface expression was assessed by flow cytometry. Surface expression of PD-L1 was significantly elevated in PDS-treated BMDCs compared to vehicle, about 3.6-fold in cDC1s and 4.7-fold in cDC2s (Figure 5.4 D, E). Together, these data show that G4 stabilisation by PDS induces PD-L1 gene, protein and surface expression.

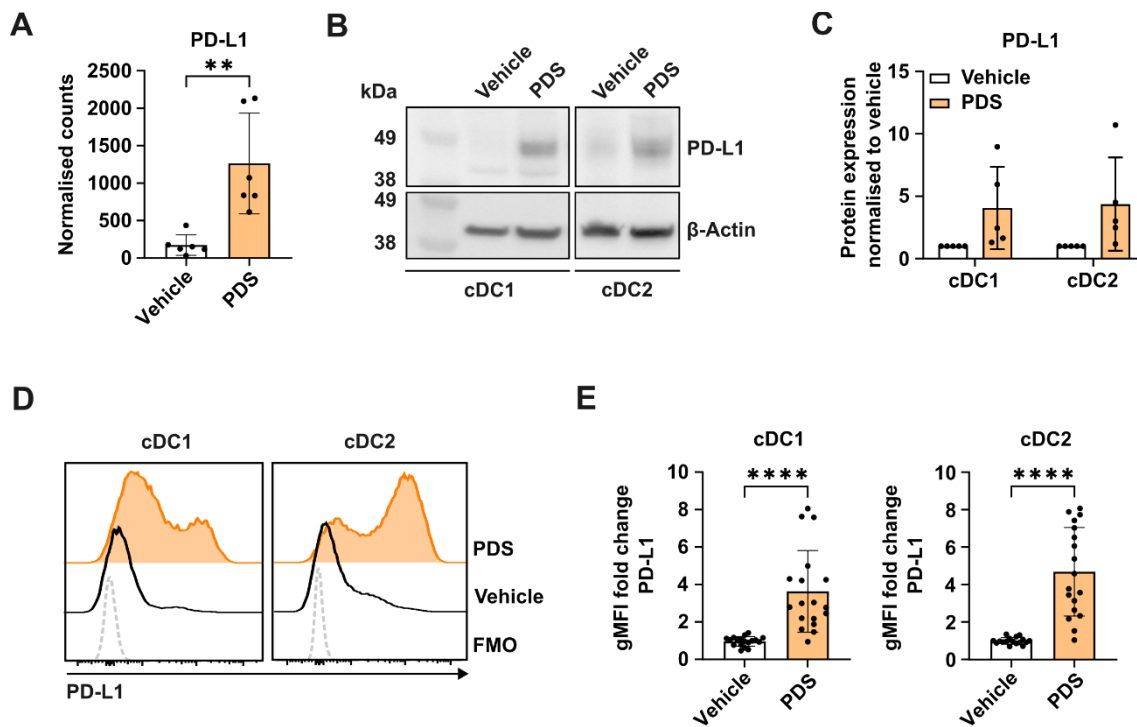


Figure 5.4 | G4 stabilisation by PDS induces PD-L1 gene, protein and surface expression in BMDCs. Murine stem cells were differentiated into immature BMDCs for seven days in presence of FMS-like tyrosine kinase 3 ligand (FLT3L). Immature BMDCs were treated with H₂O (vehicle) or 10 μM PDS for 24 h. A) Normalised PD-L1 transcript counts of vehicle- and PDS-treated BMDCs. Total RNA was isolated and subjected to 3' mRNA NGS. Data are represented as mean ± SD of n = 3 independent experiments. B) Representative western blot showing PD-L1 protein (~ 48 kDa) in vehicle- and PDS-treated BMDCs, subdivided into cDC1s (left) and cDC2s (right). GAPDH (38 kDa) was used as loading control. C) PD-L1 protein expression in cDC1s and cDC2s following PDS treatment depicted as fold change to vehicle-treated cells. Fold change was determined by normalising PD-L1 signal intensity over GAPDH (loading control) and further over vehicle-treated cells. Data are represented as mean ± SD of n = 5 independent experiments. D) Representative histogram showing the proportion of PD-L1⁺ cells in vehicle- and PDS-treated BMDCs, divided into cDC1s and cDC2s. E) Surface expression of PD-L1 on cDC1 (left) and cDC2 (right) BMDCs following treatment with PDS. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean ± SD of n = 6 independent experiments. Significance was calculated using single student's t-test (A, E) and multiple student's t-tests corrected for multiple comparisons using the Holm-Šidák method (C). ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

5.2.4 PDS induces the expression and activation of IRF1 and STAT3

The upregulation of PD-L1 gene, protein and surface expression in BMDCs following G4 stabilisation highlights the importance of understanding the mechanism underlying G4-mediated PD-L1 expression. To further characterise the molecular mechanism of how G4 formation induces PD-L1 expression, I aimed to identify proteins that might interact with the G4 structure in the PD-L1 promoter. Proteins involved in PD-L1 gene regulation include TFs such as Myc, HIF1 α , c-Jun, NF κ B, IRF1, STAT1 and STAT3 (Zerdes et al., 2018).

3' mRNA NGS revealed that of these TFs, STAT1, IRF1, JUN and STAT3 are significantly upregulated (adjusted p-value < 0.05, log₂ FC > 0.58) in PDS-treated BMDCs compared to vehicle-treated BMDCs (Figure 5.5 A). STAT1 and STAT3 are both TFs that target various genes involved in the cell cycle, cell differentiation, cell survival and immune responses (Catlett-Falcone et al., 1999; Dimberg et al., 2003, 2000; Dupuis et al., 2003; Kortylewski et al., 2005, p. 1; Lee et al., 2000). Therefore, to investigate their involvement in G4-mediated PD-L1 expression is of great interest.

STAT1 is mainly activated by interferons, but can additionally be activated by other cytokines, including IL-2, IL-6, epidermal growth factor (EGF) and tumour necrosis factor (TNF) via the JAK/STAT pathway (Hu et al., 2021). Briefly, the binding of cytokines to their respective receptors results in activation of the receptor-associated JAK1 and JAK2 protein-tyrosine kinases which subsequently phosphorylate and activate STAT1. Following activation, STAT1 is translocated to the nucleus, where it binds to gamma-activated sequences (GAS) in gene promoters to activate the transcription of target genes. However, no active binding of STAT1 to the PD-L1 promoter has been demonstrated so far (Garcia-Diaz et al., 2019). STAT1 induces the expression of many ISGs, including its downstream effector IRF1. IRF1 has been reported to play a crucial role as TF in both constitutive and IFN γ -mediated PD-L1 upregulation since inhibition of IRF1 activity or expression as well as the abrogation of the IRF1 binding site in the PD-L1 promoter resulted in decreased PD-L1 levels (Garcia-Diaz et al., 2019; Lee et al., 2006). Furthermore, the IRF1 binding site has been reported to be enriched

at endogenous G4s in human chromatin, including potential G4 control sites in promoters (Spiegel et al., 2021). Based on these data, it is likely that G4 formation in the PD-L1 promoter through PDS-mediated stabilisation leads to recruitment and binding of IRF1 to its G4-associated binding site, resulting in upregulated PD-L1 expression.

STAT3 is activated by several cytokines (IL-6, IL-10, IL-12, IL-17, IL-21), growth factors (EGF, PDGF, GM-CSF, TGF- α), carcinogens and other stress factors (LPS, adrenaline, nicotine, leptin, bile acid) (Aggarwal et al., 2009; Leaman et al., 1996). STAT3 activation via JAK/STAT pathway signalling leads to STAT3 phosphorylation and translocation to the nucleus, where it binds to the target gene promoter and induces gene expression (Aggarwal et al., 2009). In contrast to IRF1, STAT3 binding to G4 structures has not been demonstrated so far. However, it has been reported that human STAT3 contains confirmed G4 structures in its promoter that stimulate STAT3 expression (Lin et al., 2016).

IRF1 and STAT3 represent crucial targets that could play an important role in G4-mediated PD-L1 induction. Since both TFs were significantly affected by PDS on the transcriptional level, I further evaluated changes on the protein level. IRF1 protein expression was upregulated in PDS-treated BMDCs (cDC1: 1.6-fold, cDC2: 1.8-fold) (Figure 5.5 B, C). Furthermore, also STAT3 protein level was increased in cDC1s (1.7-fold) and cDC2s (2-fold) following PDS treatment (Figure 5.5 D, E). In addition, STAT3 activation was significantly elevated by PDS in cDC1s (6-fold) and induced in cDC2s (1.5-fold), indicated by the detection of Tyr705 phosphorylated STAT3 (Figure 5.5 D, E). Together, these data show that G4 stabilisation by PDS impacts IRF1 and STAT3 on the transcriptional and translational level.

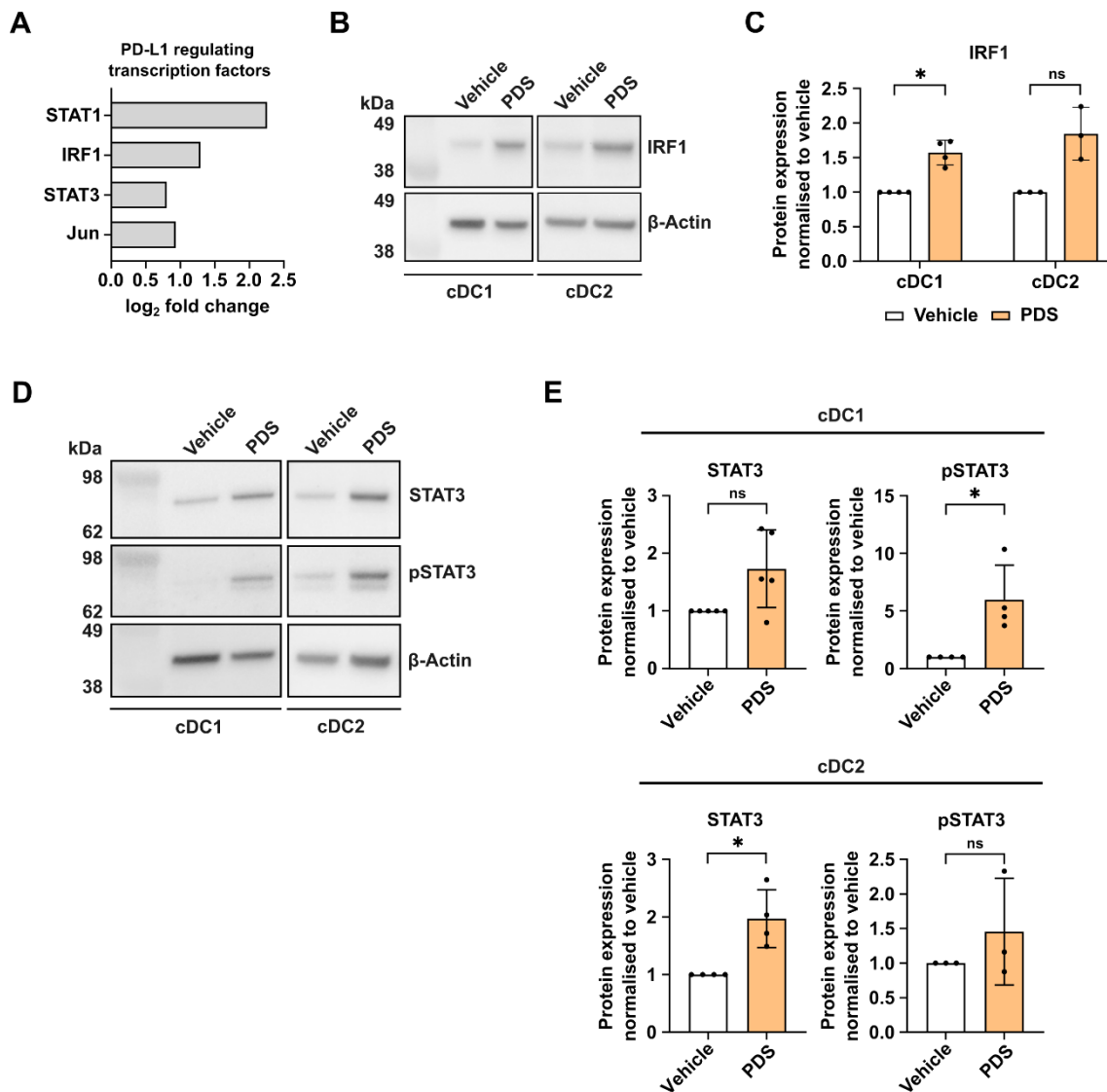


Figure 5.5 | PDS induces IRF1 and STAT3 gene and protein expression.

Murine stem cells were differentiated into immature BMDCs for seven days in presence of FMS-like tyrosine kinase 3 ligand (FLT3L). Immature BMDCs were treated with H₂O (vehicle) or 10 μ M PDS for 24 h. A) PD-L1 regulating TFs enriched in PDS-treated BMDCs according to adj. p-value < 0.05. Total RNA was isolated and subjected to 3' mRNA NGS. Data represents the log₂ FC. B+D) Representative western blots showing IRF1 (~ 49 kDa) (B), STAT3 and pSTAT3 protein (~ 86 kDa) (D) in vehicle- and PDS-treated BMDCs, subdivided into cDC1s (left) and cDC2s (right). β -Actin (42 kDa) was used as loading control. C + E) IRF1 (C), STAT3 and pSTAT3 protein level (E) in cDC1s and cDC2s following PDS treatment depicted as fold change to vehicle-treated cells. Fold change of was determined by normalising protein signal intensity over β -Actin (loading control) and further over vehicle-treated cells. Data are represented as mean $\pm$ SD of at least n = 3 independent experiments. Significance was calculated using multiple student's t-test corrected for multiple comparisons using the Holm-Šídák

method (C) and single student's t-tests (E). ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.2.5 PDS-mediated PD-L1 expression is not regulated by IRF1

To characterise the role of IRF1 in PDS-mediated PD-L1 regulation, I next assessed changes in the surface expression of PD-L1 following PDS treatment in BMDCs derived from IRF1 knockout (KO) mice (kindly provided by Prof. Sammy Bedoui, The Peter Doherty Institute, Melbourne, Australia). BMDCs were generated as previously described. On day 7 of differentiation, immature IRF1 KO (IRF1^{-/-}) BMDCs were treated with 10 μ M PDS for 24 h. PDS resulted in a 4.6-fold significantly elevated PD-L1 expression in wild-type cDC1s and cDC2s (Figure 5.6 A, B). This data supported my previous findings showing that G4 stabilisation by PDS induces PD-L1 surface expression (Figure 5.4 D, E). Additionally, PD-L1 surface level was significantly upregulated on IRF1^{-/-} BMDCs treated with PDS, revealing a 4.5-fold increase in cDC1s and 3.5-fold in cDC2s (Figure 5.6 A, B). Here, no differences in PD-L1 expression were observed between wild-type and IRF1^{-/-} cDC1s following PDS treatment. In cDC2s, PD-L1 was slightly reduced in PDS-treated IRF1^{-/-} cells compared to wild-type. However, this effect was not significant. Together, these data indicate that IRF1 is not involved in G4-mediated PD-L1 expression.

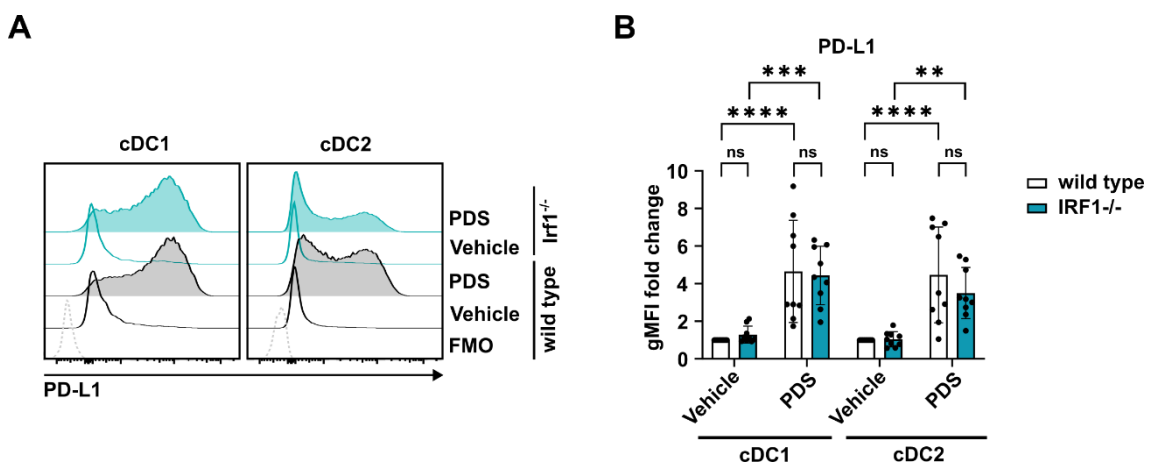


Figure 5.6 | IRF1 is not involved in G4-mediated PD-L1 expression. Stem cells isolated from IRF1 KO mice were differentiated into BMDCs in presence of FMS-like tyrosine kinase 3 ligand (FLT3L). On day 7 of differentiation, immature BMDCs were treated with H₂O (vehicle) or 10 μ M PDS. Changes in PD-L1

surface expression were assessed by flow cytometry. A) Representative histogram showing the proportion of PD-L1⁺ cells in vehicle- and PDS-treated BMDCs of wildtype and IRF1^{-/-} mice, divided into cDC1s and cDC2s. FMO: fluorescence minus one control. B) Surface expression of PD-L1 on cDC1 and cDC2s of wildtype and IRF1^{-/-} mice following treatment with PDS treatment. Differences in surface expression are depicted as geometric mean fluorescent intensity (gMFI) fold change compared to vehicle. Data are represented as mean $\pm$ SD of n = 3 independent experiments. Significance was calculated using two-way ANOVA with Fishers LSD. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

5.2.6 PDS-mediated PD-L1 expression is regulated by STAT3

As my previous results suggest that IRF1 is not involved in the mechanism of G4-mediated PD-L1 expression by using IRF1^{-/-} mice, I next investigated the role of STAT3 in PDS-induced PD-L1 expression. It has been reported that a systemic STAT3 KO (STAT3^{-/-}) causes embryonic lethality in mice (Takeda et al., 1997). Therefore, STAT3^{-/-} was induced *in vitro* by transfecting STAT3 guide RNA (gRNA) into Cas9-expressing stem cells via nucleofection. Here, two different gRNAs targeting STAT3 exon 4 (STAT3 gRNA #1) and exon 5 (STAT3 gRNA #2) were used. As a control for PD-L1 downregulation, stem cells were transfected with a PD-L1 gRNA to generate a PD-L1^{-/-}. Stem cells were differentiated into immature BMDCs for seven days in the presence of 200 ng/mL FLT3L as previously described (Figure 3.1 A). On day 7 of differentiation, immature BMDCs were treated with 10 μ M PDS for 24 h. To characterise the role of STAT3 in PDS-dependent PD-L1 expression, I assessed changes in surface expression of PD-L1 following PDS treatment in wild-type and STAT3^{-/-} BMDCs by flow cytometry. As expected, non-nucleofected BMDCs treated with PDS revealed a significant increase in PD-L1 expression compared to vehicle-treated BMDCs (Figure 5.7 A, B). Importantly, in PDS-treated STAT3^{-/-} cells, PD-L1 expression was significantly reduced in comparison to PDS-treated non-nucleofected BMDCs to a similar level as observed on PD-L1^{-/-} cells (Figure 5.7 A, B).

Moreover, a comparable effect was observed using a STAT3 knockdown (STAT3 KD) model. Here, wild-type BMDCs were generated and treated with 10 μ M PDS as previously described. In addition, 6 h prior to PDS treatment, BMDCs were

Figure 5.7 | G4-mediated PD-L1 expression is dependent on STAT3. The role of STAT3 in PDS-dependent PD-L1 expression was characterised in STAT3^{-/-} and STAT3 KD cells. STAT3^{-/-} cells were generated by transfecting STAT3 gRNA into Cas9 expressing stem cells. Stem cells were differentiated into immature BMDCs for seven days in presence of FMS-like tyrosine kinase 3 ligand (FLT3L). On day 7 of differentiation, immature BMDCs were treated with H₂O (vehicle) or 10 μM PDS for 24 h. For STAT3 KD, differentiated BMDCs were treated with 25 μM of STAT3 inhibitor S3I-201, 6 h prior to PDS treatment. Changes in PD-L1 surface expression were assessed by flow cytometry. A) Representative histogram showing the proportion of PD-L1⁺ cells in non-nucleofected, STAT3^{-/-} and PD-L1^{-/-} BMDCs (cDC1s and cDC2s) in vehicle- and PDS-treated conditions. B)

Geometric mean fluorescent intensity (gMFI) of PD-L1 in non-nucleofected, STAT3^{-/-} and PD-L1^{-/-} BMDCs (cDC1 (left) and cDC2 (right)) following PDS treatment. Data are represented as mean $\pm$ SD of n = 2 independent experiments. C) Representative histogram showing the proportion of PD-L1⁺ cells in vehicle- and PDS-treated wildtype BMDCs and STAT3 KD BMDCs (cDC1s and cDC2s) treated with PDS. D) Surface expression of PD-L1 on wildtype and STAT3 KD BMDCs (cDC1 and cDC2) following PDS treatment. Differences in surface expression are depicted as gMFI fold change compared to vehicle. Data are represented as mean $\pm$ SD of n = 2 independent experiments. Significance was calculated using Two-way ANOVA with Dunnett's multiple comparisons test. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. FMO: fluorescence minus one control.

5.2.7 STAT3 binds to the PD-L1 promoter G4 *in vitro*

To identify, if STAT3 associates with the PD-L1 promoter G4, I performed a pulldown assay using biotinylated oligonucleotides composed of the PD-L1 G4 sequence or the scrambled control (Table 15). Oligonucleotides were incubated with IFN γ -stimulated MuTu DC1940 cell lysate, as IFN γ induces STAT3 expression, thus resulting in increased STAT3 levels in the cell lysate. STAT3 binding to the PD-L1 promoter G4 was assessed by western blot. A strong signal for STAT3 (90 kDa) was detected with the PD-L1 promoter G4 oligonucleotide, while only a weak signal for STAT3 was detected with the scrambled control (Figure 5.8 A). This data indicates a specific binding of STAT3 to the PD-L1 promoter G4. In addition, I observed that activated STAT3 (pSTAT) binds to the PD-L1 G4 (Figure 5.8 A).

I further verified the binding of STAT3 to the PD-L1 promoter G4 by EMSA. The EMSA was performed using digoxigenin (DIG)-labelled oligonucleotides composed of the PD-L1 promoter G4 sequence and the STAT3 and STAT1 binding sequence (Table 16). DIG-labelled oligonucleotides were incubated with IFN γ -stimulated DC lysate. First, Thioflavin T staining of the non-denaturing gel revealed a fluorescent signal for the PD-L1 G4 oligonucleotide, confirming the formation of the PD-L1 G4 structure (Figure 5.8 B). Incubation of the PD-L1 G4 probe with lysate displayed several shifted bands, demonstrating complex formations of the G4 oligonucleotide with various proteins. A prominent complex

formed with the PD-L1 G4 oligonucleotide was shifted to the same size compared to the DNA:protein complex formed with the STAT1- or STAT3 binding sequence (Figure 5.8 B). This indicates that STAT1 or STAT3 might bind to the PD-L1 promoter G4 sequence. Incubation of the DIG-labelled PD-L1 G4 oligonucleotide with an unlabelled STAT1 or STAT3 competitor resulted in a reduction in the signal intensity of the shifted band (Figure 5.8 C). Signal intensity quantification of the shifted band revealed a 17 % reduction in the bound fraction, when incubated with the STAT3-specific competitor (Figure 5.8 D), confirming STAT3 binding to the PD-L1 promoter G4. In addition, the bound fraction was reduced by about 45 % following incubation with the STAT1-specific competitor, demonstrating that STAT1 also binds to the PD-L1 promoter G4, even with higher affinity than STAT3. Interestingly, incubation of the PD-L1 promoter G4 oligonucleotide with both, STAT1 and STAT3 competitors, resulted in a 60 % reduction of the bound fraction (Figure 5.8 D).

Altogether, the pulldown assay and the EMSA, both demonstrated the association of STAT3 with the PD-L1 promoter G4 *in vitro*.

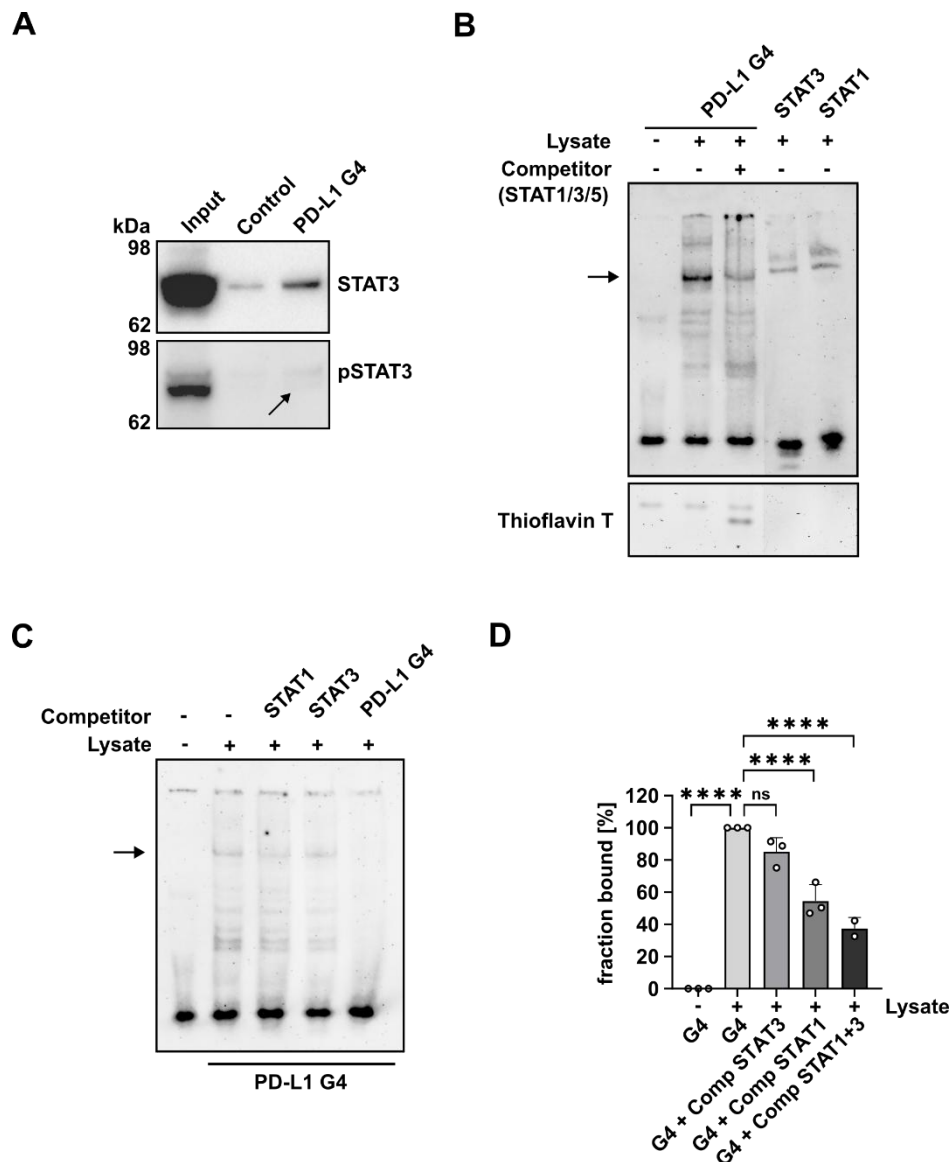


Figure 5.8 | STAT3 associates with the PD-L1 promoter G4. STAT3 binding to the PD-L1 promoter G4 structure was assessed by pulldown assay and EMSA. Pulldown assay was performed using 3' biotinylated oligonucleotides composed of the PD-L1 promoter G4 sequence and the scrambled control. A) Representative western blot displaying the binding of STAT3 (90 kDa) and pSTAT3 to the PD-L1 promoter G4 and the scrambled control. B) Above: Representative EMSA displaying Protein:DNA complexes formed following incubation of the DIG-labelled PD-L1 promoter G4, STAT3- and STAT1-binding sequences with IFN γ -stimulated cell lysate. Below: Thioflavin T staining detecting G4 structure formation. C) Representative EMSA showing formed Protein:DNA complexes following incubation of the DIG-labelled PD-L1 promoter G4 sequence with IFN γ -stimulated cell lysate and unlabelled competitors. D) Percentage of bound fraction. Data are represented as mean $\pm$ SD of at least $n = 2$ independent experiments. Significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3 Discussion

In the previous chapter, I showed that G4 stabilisation by PDS induces transcriptional reprogramming in DCs by stimulating the expression of several genes. To further understand how stabilised G4 structures contribute to changes in gene expression, I investigated the mechanisms underlying G4-mediated transcriptional stimulation by the example of PD-L1.

5.3.1 PDS-induced PD-L1 expression is due to a G4 motif located in the PD-L1 gene promoter

Transcriptome-wide analysis showed a significant increase in PD-L1 gene expression following PDS treatment (Figure 5.4 A). Several studies described an abundance of G4 structures in gene promoters associated with the regulation of gene expression (Chen et al., 2024; Lago et al., 2021; Li et al., 2023; Shen et al., 2021; Hänsel-Hertsch et al., 2016). Initial analysis demonstrated that G4s represent a physical obstacle to RNA polymerase II, thereby inhibiting transcription (Bejugam et al., 2007; Cogoi and Xodo, 2006; Morgan et al., 2016; Siddiqui-Jain et al., 2002). However, accumulating evidence indicates a correlation between G4s and high gene expression, which is dependent on several factors, including the strand harbouring the G4 motif, recruitment of TFs and chromatin remodelling (Agarwal et al., 2014; Hou et al., 2019; Lago et al., 2021; Mao et al., 2018). Genome-wide mapping of DNA-G4 structures revealed a putative G4 motif in the promoter of the PD-L1 gene in the mouse genome, which might form a G4 structure under G4 stabilising conditions (Marsico et al., 2019). Here, I showed that the PD-L1 promoter G4 adopts a G4 structure *in vitro* and *in cellulo* by ThT staining, CD and ChIP (Figure 5.2). Interestingly, the fraction of recovery (% IP) of PD-L1 G4 DNA was lower in PDS-treated cells compared to untreated cells (Figure 5.2 C). This observation was contrary to expectations, as I assumed that G4 stabilisation by PDS would result in an increased recovery of PD-L1 G4 DNA. Hence, these data suggest that PDS might interfere with the binding of BG4 to the G4 structure, thereby pulling down less G4 DNA. Recently, the group of Marco Di Antonio reported that G4 ligands can reduce protein

accessibility at G4 structures using BG4 (Nuccio et al., 2024). This observation suggests that a reduction in BG4 binding associated with a lower IP recovery rate, rather than an increase, is indicative of G4 engagement by PDS. Using a Luciferase Reporter Assay, I demonstrated that the PD-L1 promoter G4 motif positively affects gene expression compared to a scrambled control and a mutated G4 motif (Figure 5.3). Nevertheless, this was just an artificial approach. The removal or mutation of the G4 motif in the PD-L1 promoter sequence *in cellulo* would have strengthened this conclusion, however the precise mutation of the G4 motif *in cellulo* would have been tricky due to the presence of multiple G-tracts in the sequence (Figure 5.1). Further experiments, including G4-destabilizing compounds (Mitteaux et al., 2021) as additional control could support the importance of the G4 motif in the PD-L1 promoter sequence for PD-L1 expression.

5.3.2 STAT1 and STAT3 might play a role in G4-mediated PD-L1 expression

Computational algorithms identified that TF binding sites are often close to or overlapping with G4 motifs in human gene promoters (Hou et al., 2019; Spiegel et al., 2021). Multiple studies demonstrated that G4s in promoters can bind TFs, resulting in elevated gene expression (Lago et al., 2021; Niu et al., 2019; Raiber et al., 2012; Spiegel et al., 2021). PD-L1 gene expression can be regulated by several TF, including Myc, HIF1 α , NF κ B, IRF1, STAT1 and STAT3 (Bellucci et al., 2015; Casey et al., 2016; Gowrishankar et al., 2015; Lee et al., 2006; Marzec et al., 2008; Noman et al., 2014). I have chosen STAT1 and STAT3 for further analysis, as both TFs target various genes involved in the cell cycle, cell differentiation, cell survival and immune responses (Catlett-Falcone et al., 1999; Dimberg et al., 2003, 2000; Dupuis et al., 2003; Kortylewski et al., 2005, p. 1; Lee et al., 2000). A mechanism involving STAT1 and/or STAT3 in G4-mediated stimulation of PD-L1 expression might be applicable to several other genes observed to be differentially expressed in DCs following PDS stimulation. STAT1 is activated via the JAK/STAT pathway and induces, among others, the

expression of its downstream effector IRF1, a prominent TF involved in PD-L1 expression (Garcia-Diaz et al., 2019; Lee et al., 2006). The IRF1 binding site is enriched at endogenous G4s in human chromatin, including potential G4 sites in promoters (Spiegel et al., 2021). Therefore, IRF1 represented a potential candidate responsible for G4-mediated PD-L1 expression. However, PD-L1 expression induced upon PDS treatment was not affected in IRF1^{-/-} cells (Figure 5.6), demonstrating that IRF1 is not involved in G4-mediated PD-L1 regulation. This could be likely because the proposed IRF1 binding profile in mice (GAAACTGAAA, JASPAR database) was not overlapping with or in close proximity to the identified G4 structure in the PD-L1 gene promoter. The role of STAT1 in regulating the expression of PD-L1 was exclusively described through the induction of IRF1 (Garcia-Diaz et al., 2019; Lee et al., 2006). To date, active binding of STAT1 to the PD-L1 promoter has not been detected. Here, for the first time, I showed that STAT1 binds to a G4 structure *in vitro*, the PD-L1 promoter G4 (Figure 5.8 C, D), demonstrating that STAT1 can directly bind to the PD-L1 promoter through association with these secondary structures. Nevertheless, the binding of STAT1 to the G4 structure *in cellulo* as well as its importance for G4-induced PD-L1 expression still needs to be explored in the future.

Human STAT3 contains putative G-quadruplex sequences in its gene promoter, which were confirmed to form G4 structures that induce STAT3 expression (Lin et al., 2016). Consequently, STAT3 expression was significantly increased under G4 stabilising conditions in murine BMDCs (Figure 5.5 A, E), which might be due to several potential G4 motifs mapped to the gene promoter of mouse STAT3 (Marsico et al., 2019). Furthermore, PDS induced STAT3 activation (Figure 5.5 E). Previous studies described the use of G4 forming-aptamers for specific inhibition of STAT3 expression in cancer cells (Esposito et al., 2023; Ogloblina et al., 2018). However, a mechanistic link between G4 structures and STAT3 activation is missing. STAT3 is mainly activated in response to IL-6 signalling (Park et al., 2004; Zhong et al., 1994). Transcriptome analysis demonstrated a significant increase in IL-6 expression in BMDCs following PDS treatment (Figure 3.9 A). An IL-6 feedback loop caused by PDS-induced IL-6 expression followed

by potential binding of IL-6 to its receptor on the DC surface could be the reason for increased STAT3 phosphorylation upon PDS stimulation.

In this study, STAT3 was identified to play an important role in PDS-mediated PD-L1 expression. Loss or inhibition of STAT3 resulted in a significantly reduced PD-L1 expression following PDS treatment (Figure 5.7). In contrast to STAT1, activated STAT3 (pSTAT3) directly binds to the PD-L1 promoter (Tong et al., 2020). However, STAT3 binding to G4 structures has not been shown up to now. A potential STAT3 binding site has been identified in close proximity (~ 100 bp downstream) to the PD-L1 promoter G4. Furthermore, I showed that STAT3 binds to the PD-L1 promoter G4 *in vitro* (Figure 5.8 C, D). Nevertheless, the binding of STAT3 to the PD-L1 promoter G4 *in cellulo* still needs to be confirmed by ChIP. Furthermore, mapping of STAT3 binding to G4 structures in DCs before and after PDS treatment could additionally give insights whether PDS binding to the PD-L1 G4 structure promotes STAT3 attachment to the promoter to boost PD-L1 expression. However, a recent study by Nuccio et. al. (2024) demonstrated that BG4 Cut&Tag following PDS treatment is inconsistent, as the ligand can differentially interfere with local protein binding, resulting in either increased or decreased protein accessibility to the G4 structure. Additionally, they showed that structurally distinct ligands induce various responses at the same G4 site, indicating that G4 ligands differentially impact individual proteins. Therefore, STAT3 ChIP or STAT3 Cut&Tag with untreated and PDS-treated cells followed by PD-L1 qPCR provides an alternative opportunity to investigate whether G4 stabilisation by PDS increases STAT3 binding to the PD-L1 promoter.

Analysis of PD-L1 promoter G4:protein interaction by EMSA revealed that both STAT1 and STAT3 can bind the PD-L1 G4, suggesting a possible synergistic role of both TFs in G4-mediated PD-L1 expression. However, it remains unclear, if they bind to the G4 structure individually as homodimer or in the form of a STAT1/3 heterodimer. Multiple studies have shown that STAT1 and STAT3 can form heterodimers, especially upon IL-6 stimulation (Haan et al., 2005; Weis et al., 2009; Zhong et al., 1994). Furthermore, Sasidharan Nair et al. (2018) demonstrated that STAT1 and STAT3 form an active heterodimer, which binds to

the PD-L1 promoter to drive its gene expression. Hence, analysis of PD-L1 G4:protein interaction using STAT1 and STAT3 recombinant homo- and heterodimer proteins could provide insights into the binding mode of STAT1 and STAT3 to the PD-L1 promoter G4 and would further support the synergistic effect of both TFs in G4-induced PD-L1 expression.

Recruitment of TFs to G4 structures in promoters can induce gene expression in two different ways: either TF binding to promoter G4s leads to the recruitment of RNA polymerase II (Spiegel et al., 2021) or gene transcription is enhanced by TF-mediated G4 unfolding (David et al., 2019). The mechanism underlying STAT3 and or STAT1-dependent PD-L1 gene expression following binding to the PD-L1 promoter G4 remains unknown and needs to be explored in future experiments including RNA polymerase II-ChIP and CD or NMR to determine G4 structure unfolding in the presence and absence of the respective TFs.

Besides several inflammatory stimuli, PD-L1 expression can be upregulated in response to DNA DSB in cancer cells. DSB formation caused by ionising radiation resulted in the activation of STAT1/STAT3 and IRF1, which are responsible for DSB-dependent PD-L1 upregulation (Sato et al., 2017). It is already known that PDS leads to G4-dependent DNA DSB formation (Rodriguez et al., 2012; Zimmer et al., 2016). Hence, apart from a direct effect of G4 structure formation on PD-L1 expression through G4 stabilisation within the PD-L1 promoter, PD-L1 upregulation in DCs following PDS stimulation could be likely due to an indirect effect of G4s, caused by G4 stabilisation-induced DSB formation and activation of STAT1/STAT3 and IRF1 pathway.

In conclusion, based on the previous findings, I proposed the following current model of G4-mediated PD-L1 expression in DCs (Figure 5.9). G4 stabilisation by PDS leads to G4 structure formation in the PD-L1 gene promoter. PDS treatment results in transcriptional changes of several genes, including increased STAT3 levels (due to G4 formation in STAT3 gene promoter) and STAT3 activation. Increased STAT3 activation can be attributed to induced IL-6 expression, which in turn leads to the activation of the JAK/STAT pathway. JAK/STAT signalling

causes phosphorylation and activation of STAT1 and STAT3, their formation of homo-or heterodimer and translocation to the nucleus. Within the nucleus, these proteins bind to the G4 structure in the PD-L1 promoter, thus inducing PD-L1 gene expression, which further results in increased PD-L1 protein and surface levels. Several genes affected through G4 stabilisation by PDS associated with DC activation and maturation were identified as target genes of STAT1 and STAT3 (identified using TFLink (Liska et al., 2022)), which additionally contain potential G4 motifs in their gene promoter. Therefore, the proposed mechanism underlying G4-mediated PD-L1 regulation involving the binding of STAT1 and STAT3 might be applicable to multiple additional genes that are impacted by G4 stabilisation in DCs, providing a potential explanation of how G4 structure-induced transcriptional reprogramming is regulated, resulting in phenotypical and functional changes of DCs.

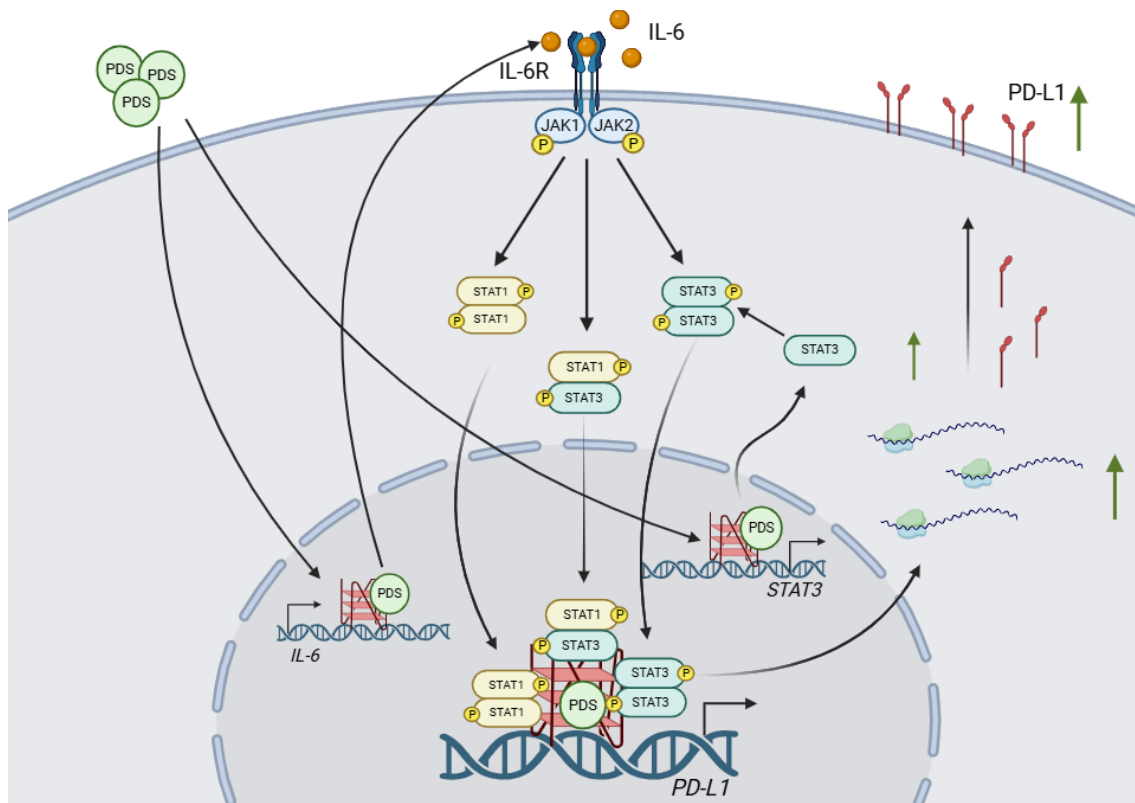


Figure 5.9 | Current model of G4-mediated PD-L1 expression under PDS-stabilising conditions. G4 stabilisation by PDS leads to induced STAT3 expression and activation. STAT3 can be activated by IL-6 through the JAK/STAT pathway. Briefly, IL-6 binding to its receptor IL-6R leads to activation of JAK1/2 which further induce STAT1 and STAT3 phosphorylation and activation. STAT1 and STAT3 form homo- or heterodimers and translocate to the nucleus, where they act as a transcription factor for PD-L1 regulation. Under G4 stabilising conditions, STAT1 and/or STAT3 bind to the G4 structure identified in the PD-L1 promoter region, resulting in upregulated PD-L1 expression.

6. Final discussion

Dendritic cells are the most potent antigen-presenting cells that link innate and adaptive immunity by sensing invading pathogens or damaged cells and inducing an antigen-specific immune response (Steinman, 2006). Therefore, DCs play a critical role in various clinical implications, such as anti-viral and anti-tumour immunity (Broz et al., 2014; Domenjo-Vila et al., 2023; Ferris et al., 2020; Hildner et al., 2008; Nopora et al., 2012; Sánchez-Paulete et al., 2016; Spranger et al., 2017). Current immunotherapeutic strategies focus on enhancing immune responses towards cancer or virus-infected cells e.g. by blocking inhibitory immune checkpoint molecules on the surface of cancer and immune cells (Blomberg et al., 2023; Chen et al., 2021; Roy et al., 2018). Further approaches boosting immune responses include adoptive cell transfer, therapeutic vaccines and the use of immune system modulators (e.g. cytokines) (Igarashi and Sasada, 2020; Mirtaleb et al., 2021; Němečková and Roubalová, 2019; Rosenberg, 2014; Rosenberg and Restifo, 2015). Dendritic cells have been reported to be important for the efficacy of some anti-tumour therapies (Peng et al., 2020; Salmon et al., 2016; Sánchez-Paulete et al., 2016). As key activators of immune responses, DCs themselves provide a promising target for immunotherapeutic approaches. One of the most popular strategies is the adoptive transfer of activated TAA-loaded DCs (Gu et al., 2020). Within the TME, DCs are impeded in activation, maturation and their function to initiate an anti-tumour response due to immunosuppressive regulators (Xiao et al., 2023). Therefore, several strategies based on targeting DCs focus on the potential to enhance DC activation. Such approaches include administration of immune-enhancers, like FLT3L, GM-CSF and CpG ODN and fusion of tumour antigens to antibodies targeted against DC-specific surface receptors to support transport of antigens to DCs (Bhardwaj et al., 2020; Dhodapkar et al., 2014; Masterman et al., 2020; Oosterling et al., 2006; Sluijter et al., 2015). While these approaches rely on extrinsic stimulation of DCs, intrinsic programming of specific target genes involved in cellular pathways could provide a potential new strategy to specifically regulate DC activation.

In the past years, DNA secondary structures, such as G-quadruplexes, have gained great attention as a potential therapeutic target. Due to their enrichment in regions associated with genome regulation, G4s are implicated in the regulation of biological processes, such as telomere maintenance, replication, transcription and translation (Bochman et al., 2012; Rhodes and Lipps, 2015). The importance of G4 structures as an attractive target for therapeutic approaches in multiple diseases became apparent by the use of G4-binding molecules (G4 ligands), which specifically bind to and stabilise the formation of G4s in the genome. To date, the use of G4 ligands has shown a substantial therapeutic potential against cancer, neurological disorders and viral infections by impeding processes important for disease biology (Artusi et al., 2021; Burger et al., 2005; Gowan et al., 2002; Liu et al., 2022; Miyazaki et al., 2012; Paul et al., 2020; Perrone et al., 2014; Shioda et al., 2018; Simone et al., 2018; Zimmer et al., 2016). These studies focus on the direct impact of G4s on the target cells or pathogens, aiming to improve disease outcomes. However, therapeutic approaches focusing on the potential of G4 stabilisation in initiating/boosting an appropriate immune response have not been investigated so far.

G4 structures and their role in biological processes have already been extensively studied in various cell types, including stem cells, cancer cell lines and neurons (Baser et al., 2023; Dutta et al., 2021; Revikumar et al., 2022; Richl et al., 2024; Tabor et al., 2021; Zyner et al., 2022). However, little is known about the effect of G4s in immune cells. Due to their critical role in anti-viral and anti-tumour immunity, in this thesis, I aimed to investigate the impact of G4 formation on cellular changes in DCs and their function in T cell priming to gain a better understanding of whether DCs can be regulated on the genomic level to improve the induction of T cell-mediated immune responses.

6.1 G4-stabilisation in DCs as an immunotherapeutic strategy for cancer and viral infections

In this thesis, I showed that G4 stabilisation using the well-known G4 ligand PDS induces activation and maturation of conventional DCs (cDC1 and cDC2) *in vitro* by increasing surface expression of co-stimulatory (CD40, CD80, CD86) and MHC class molecules as well as enhancing the migratory capacity. Expression of some DC markers, such as CD86 and MHC II were increased to a higher extent on cDC2s compared to cDC1s following PDS administration, demonstrating that G4 stabilisation regulates a stronger response in cDC2s. In line with this, PDS treatment of DCs enhanced their capability of T cell priming, especially the activation of CD4⁺ T cells. cDC1s play a key role in regulating CTL and T_h1 responses to intracellular pathogens and tumour cells by cross-presenting exogenous antigens and TAAs on MHC I to naïve CD8⁺ T cells and priming CD4⁺ differentiation to T_h1 helper cells (Ashour et al., 2020; Bedoui et al., 2009; Ferris et al., 2020; Hildner et al., 2008; Svensson-Arvelund et al., 2022). cDC2s are rather destined for the induction of T_h2 and T_h17 immune responses to eliminate extracellular pathogens and parasites, by presenting antigens on MHC II to CD4⁺ T cells (Kumamoto et al., 2013; Schlitzer et al., 2013). In the past years, the role of cDC2s in driving tumour immunity has gained importance. cDC2s present TAAs to CD4⁺ T cells (Ruhland et al., 2020), which plays a critical role in tumour immunosurveillance by supporting effective CD8⁺ T cell priming (Bos and Sherman, 2010; Ferris et al., 2020). Similar to anti-viral immunity (Christensen et al., 1999; Jellison et al., 2005), cytotoxic CD4⁺ T cells can also directly mediate cancer cell killing (Cachot et al., 2021; Haabeth et al., 2018; Oliveira et al., 2022; Xie et al., 2010). Due to the crucial role of DCs in T cell-mediated immunity, G4 stabilisation in DCs provides a potential strategy to prime DC activation to achieve advanced T cell immunity against viral infections and cancer. Especially within the TME, DC activity is suppressed by inhibitory immune regulators (Elia et al., 2008; Gabrilovich et al., 1996; Gottfried et al., 2006; Hangai et al., 2016; Suthen et al., 2022). Therefore, G4 targeting in DCs might be a promising approach to overcome these immunosuppressive effects in the TME to induce an efficient immune response. In this study, I showed that intratumoral injection of PDS

results in a significant upregulation of co-stimulatory molecules (CD40, CD80 and CD86) on cDC1 and cDC2 DCs in B16OVA melanoma, demonstrating that PDS triggers DC activation within the tumour. Furthermore, the cDC1 population was completely lost in PDS-administered tumours, indicating that these cells following activation leave the tumour site to migrate to dLN for T cell priming (Roberts et al., 2016). Indeed, DCs isolated from tdLNs of PDS-treated mice revealed a higher abundance of G4 structures compared to DCs in tdLNs of vehicle-treated mice, indicating the migration of DCs from the tumour site to tdLNs. Although PDS was administered locally to the tumour, there is a minor possibility that it is diffused from the tumour to LNs and stabilised G4s in LN-resident DCs. To get evidence on cDC1 migration to tdLNs following intratumoural PDS administration, future experiments should include the use of fluorescently labelled PDS and fluorescently stained tumour-derived cDC1s, which allows to track PDS and cDC1 migration and to distinguish migrated cDC1s from resident DCs within the LNs.

Intratumoural injection of PDS resulted in reduced tumour growth associated with tumour infiltration of NK cells, B cells, monocytes, T_{regs}, and CD8⁺ T cells (unpublished data by Nils Kosiol). G4 ligand-based tumour suppression has already been demonstrated in multiple studies (Fukuda et al., 2023; Hu et al., 2019; Lagah et al., 2014; Nakamura et al., 2017). A possible mechanism of PDS-mediated tumour rejection could be that intratumoural administration induces DC activation, which migrate to the tdLN to induce cytotoxic CD8⁺ T cells. CTLs in turn will then infiltrate the tumour to conduct their effector function on tumour cell killing. It needs to be mentioned that the observed DC activation might not only be based on a direct effect of PDS-mediated G4 stabilisation in DCs but rather on a systemic effect caused by PDS. G4 ligands, such as PDS, are cell permeable and thus can affect any cell present within the TME. Furthermore, PDS is already known to cause cytotoxicity by inducing DNA damage and promoting cell cycle arrest (Bossaert et al., 2021; Hou et al., 2022; Rodriguez et al., 2012). In line with this, only 5-10 % of B16OVA tumour cells were viable *in vivo* 24 h after intratumoural injection of PDS (unpublished data by Nils Kosiol).

Induction of immunogenic cell death within the tumour leads to the release of TAAs which will be captured by DCs resulting in DC activation (Woo et al., 2014). To investigate the direct effect of G4 stabilisation on DC activation and function within the TME, DC-targeted delivery of the G4 ligand is required. Several immunotherapeutic approaches based on DC targeting have already been reported. DCs can be directly stimulated *in vivo* by targeted delivery of adjuvants and antigens using antibodies coupled with TAAs or nanoparticles towards DC-specific surface receptors (Bonifaz et al., 2004; Hanlon et al., 2014; Hutten et al., 2016; Idoyaga et al., 2011; Sancho et al., 2008). Depending on the target receptors, distinct DC subsets can be specifically targeted (DEC205 and CLEC9A targets cDC1s, CLEC4A4 targets cDC2, CLEC12A targets cDCs, pDCs, MoDCs), allowing individual DC activation (Kreutz et al., 2013). Direct targeting of DCs *in vivo* using DC-specific antibodies fused with nanoparticles containing the G4 ligand and a TAA could be a possible approach to deliver the G4 ligand specifically to DCs to stimulate DC activation for improved tumour immunity. G4-mediated stimulation of DCs *in vitro* did not result in as strong mature DCs as observed following stimulation with TLR ligands, such as CpG or LPS. Therefore, a combined delivery of the G4 ligand with an additional adjuvant should be considered. Unexpectedly, co-treatment of DCs with PDS and CpG or LPS *in vitro* resulted in reduced expression of co-stimulatory molecules compared to the TLR ligands alone (data not shown). TLR4 (LPS) and TLR9 (CpG) engagement leads to the activation of TFs like AP-1 and NF- κ B via the Toll/Interleukin-1 receptor signal pathway, resulting in immune cell activation (Wagner, 2001). The AP-1 binding site has been shown to overlap with G4 structures in gene promoters (Lago et al., 2021). Furthermore, Spiegel et al. (2021) reported that PDS competes with TF binding to G4 structures. Therefore, reduced expression of co-stimulatory molecules on DCs following co-stimulation with PDS and CpG might be due to PDS interfering with AP-1 binding. Interestingly, cotreatment of DCs with PDS and IFN γ showed a significant increase in the expression of co-stimulatory and MHC class molecules, to a similar or even higher extent compared to CpG stimulation, especially on cDC2s (data not shown). IFN γ signals via the JAK/STAT pathway (Varinou et al., 2003), leading to the activation

of the downstream TF STAT1, which plays a key role in DC maturation (Jackson et al., 2004). In addition, STAT1 gene expression was significantly induced in DCs stimulated with PDS. This indicates a synergistic effect of PDS and IFN γ on DC activation, resulting in a fully mature DC. Hence, PDS combined with IFN γ represents a potential adjuvant for DC-targeted immunotherapy.

Besides direct-targeting of DCs *in vivo*, one of the most popular strategies of DC vaccines involves the *ex vivo* stimulation of DCs loaded with TAAs followed by their reinfusion into patients to carry out effector functions (Gu et al., 2020). One critical hurdle of DC-based vaccines is to overcome tumour-mediated immunosuppression. cDC2 vaccination based on naturally occurring DCs isolated from peripheral blood was observed to be more efficient in inducing anti-tumour immune responses, particularly those mediated by T_h17 cells, compared to cDC1 vaccination. This anti-tumour effect of cDC2s was dependent on CD4⁺ T cells (Laoui et al., 2016). Due to the reinforced effect of PDS-mediated G4 stabilisation in combination with IFN γ on cDC2 activation *in vitro*, cDC2 vaccination involving *ex vivo* DC stimulation via combined administration of PDS and IFN γ could enhance the efficacy of CD4⁺ T cell-mediated anti-tumour immunity. However, the half-life of G4 ligands needs to be considered regarding DC vaccine efficiency.

Transcriptome analysis of pan-DCs showed that PDS-mediated G4 stabilisation induces the expression of several anti-viral genes. pDCs are the important mediators of anti-viral immunity due to their ability to produce high amounts of type-I IFNs in response to viral infection (Asselin-Paturel et al., 2001; Cella et al., 2000; Frenz et al., 2014; Schmidt et al., 2005; Swiecki et al., 2010). Type-I IFNs derived from pDCs do not only directly inhibit viral infection, but also initiate adaptive anti-viral immunity by activating the effector function of NK cells, B cells, mDCs and T cells (Gerosa et al., 2005; Hibbert et al., 2003; Jegou et al., 2003; Kolumam et al., 2005; Le Bon et al., 2003). The direct impact of G4 stabilisation on pDC phenotype and function was beyond the scope of this thesis. However, transcriptomic analysis indicates that PDS-mediated G4 stabilisation, similar to cDCs, enhances pDC activation through the induction of several interferon-

stimulated anti-viral genes. Hence, directed pDC activation via G4 ligand-targeting could be a potential opportunity to improve anti-viral immunity. However, the impact of G4 stabilisation on pDC phenotype and function first needs to be further investigated.

6.2 The use of G4 stabilisation to reprogram DCs towards a tolerogenic phenotype in aGvHD

In this thesis, I investigated the impact of G4 ligands on aGvHD for the first time. Acute GvHD is a life-threatening complication that often occurs after allogeneic stem cell transplantation. In aGvHD, immunocompetent donor T cells recognise the host as foreign and induce an immune response resulting in host tissue and organ damage (Zeiser and Blazar, 2017). In contrast to cancer and viral infections, in which DCs are required to induce an appropriate adaptive immune response, DCs play a central role in GvHD onset by presenting host-antigens to HLA-mismatched donor-derived T cells (Duffner et al., 2004). In this study, I showed that intraperitoneal injection of PDS enhances survival and ameliorates GvHD severity in mice. However, the mechanism underlying PDS-mediated GvHD reduction remains unclear. As previously mentioned, PDS can affect any cell type due to cell permeability. Since every cell type performs individual functions, the impact of systemic PDS administration needs to be investigated on each cell type to fully understand the PDS-induced mechanism causing GvHD improvement. Furthermore, investigating the mechanism of PDS-enhanced GvHD might be even more challenging, as GvHD targets multiple tissues and organs (Glucksberg et al., 1974). Nevertheless, the effect of PDS due to G4 stabilisation on distinct immune cells involved in GvHD is mainly unknown so far. T_{reg} and myeloid suppressor cells are crucial for reducing GvHD severity (Edinger et al., 2003; Highfill et al., 2010; Nguyen et al., 2007). Furthermore, besides their important role in the initiation of GvHD onset, host and donor DCs have been described to induce donor T cell tolerance against host tissues after allo-HCT resulting in less severe GvHD (Teshima et al., 2002). Induction of DC-mediated immune tolerance correlates with an immature DC state (Hadeiba et al., 2008;

Probst et al., 2005). Resting DCs induce T cell tolerance through the interaction of PD-L1 and CD80/86 with the inhibitory molecules PD-1 and CTLA-4 expressed on T cells (Blazar et al., 2003; Probst et al., 2005). Moreover, transfer of host CCR9⁺ pDCs as well as pre-pDCs was shown to inhibit/attenuate GvHD in mice receiving MHC-mismatched donor T cells, while retaining the GVL effect (Hadeiba et al., 2008; Hassan et al., 2019; Lu et al., 2012). CCR9⁺ pDCs and pre-pDCs can promote T_{reg} expansion and function as well as suppress antigen-specific immune responses, thus providing a tolerogenic effect on the induction of donor T cells against host tissues (Hadeiba et al., 2008; Lu et al., 2012). Lu et al. (2012) proposed a feedback model, in which donor pDCs induce activation of T cells that secrete high levels of IFN γ . IFN γ production by T cells in turn induces IDO expression by donor pDCs, which initiates the generation of T_{regs} and the suppression of alloreactive T cell activity. These data indicate a novel approach for reducing GvHD by modulating DCs towards a tolerogenic phenotype. Preclinical studies described the great potential of tDCs for GvHD therapy, as tDC administration resulted in induced antigen-specific graft tolerance and long-term allograft survival (Hill et al., 2011; Yang et al., 2013). In addition, direct DC targeting *in vivo* using a histone deacetylase inhibitor led to reduced expression of co-stimulatory molecules, increased production of IDO and promoted T_{reg} expansion, resulting in attenuated GvHD in mice (Reddy et al., 2008). Transcriptome analysis revealed that PDS-mediated G4 stabilisation induces transcriptional reprogramming in BMDCs. Similar observations have already been described in neurons and cancer cell lines caused by multiple G4 ligands (Escarcega et al., 2023; Revikumar et al., 2022). Transcriptional regulation of genes is mainly associated with an abundance of G4 structures in gene promoters (Chen et al., 2024; Lago et al., 2021; C. Li et al., 2021; Li et al., 2023; Shen et al., 2021). G4 stabilisation in gene promoters has been shown to regulate gene expression positively and negatively (Chen et al., 2024; C. Li et al., 2021; Shen et al., 2021; Siddiqui-Jain et al., 2002; Morgan et al., 2016; Hänsel-Hertsch et al., 2016). Most DEGs linked to DC activation and maturation contain a G4 motif in its promoter region (Marsico et al., 2019), indicating that cellular changes in DCs upon PDS treatment can be referred to the G4-mediated regulation of

gene expression. These data demonstrate that G4 stabilisation can be used to reprogram the DC phenotype by affecting transcriptome regulation. tDCs are characterised by high expression of inhibitory molecules (e.g. PD-L1, IDO, IL-10) and low expression of co-stimulatory molecules, resulting in impaired ability of T cell activation (Morelli and Thomson, 2007). Hence, targeting G4 structures by G4 ligands in the gene promoter of inhibitory molecules, such as PD-L1 and IDO might reprogram DCs towards a tolerogenic phenotype, resulting in reduced GvHD. The approach of focusing on specific targeting of G4-containing genes is not new and has already been used as a therapeutic strategy in cancer. Here, G4 ligands caused reduced tumour growth, which correlated with a decrease in oncogene expression due to G4 stabilisation in the gene promoter (Brito et al., 2015; Calabrese et al., 2018; Felsenstein et al., 2016). However, due to the high prevalence of DNA G4 motifs in the human genome (~ 700.000) (Chambers et al., 2015), G4 ligands target G4 structures genome-wide. Therefore, selective targeting of specific G4 structures is needed to reprogram DCs to a tolerogenic phenotype. However, ligand binding to specific G4 motifs is a long-standing challenge. Several strategies for selective targeting of individual G4 DNA structures have currently been discussed. One potential strategy is based on a G4 ligand conjugated to a guide oligonucleotide that specifically directs the ligand to the targeted G4 structure (Berner et al., 2024). Another approach made use of G4 ligands fused to an inactive Cas9, which will be guided by an individual sgRNA that facilitates specific targeting of G4 structures without affecting other G4s within the genome (Qin et al., 2024). The latter represents a promising tool, as several compounds targeting G4s, including G4 unfolding helicases, can also be conjugated with the inactive Cas9 protein, which besides specific G4 stabilisation also allows targeted unwinding of G4 structures.

6.3 The role of STAT1 and STAT3 in G4-mediated reprogramming of DCs

In this thesis, I investigated the mechanism underlying G4-mediated transcriptional programming in DCs by the example of PD-L1. Among 600 DEGs, PD-L1 was significantly upregulated upon PDS stimulation in DCs. Genome-wide mapping revealed that most DEGs, including PD-L1, contain a G4 motif within the promoter region (Marsico et al., 2019). In this study, I showed that the PD-L1 promoter G4 motif can adopt a G4 structure *in vitro* and *in cellulo* and that it interacts with TFs such as STAT1 and STAT3 *in vitro*. This finding is supported by multiple previous studies, describing G4s as TF binding hubs (De Rache et al., 2024; Frasson et al., 2021; Lago et al., 2021; Spiegel et al., 2021). According to the previous data, I proposed a potential mechanism of G4-mediated PD-L1 regulation, involving a feedback loop system. Briefly, PDS induces transcriptional changes in several genes, including STAT3, STAT1 and IL-6. Induced IL-6 secretion in turn leads to the activation of the JAK/STAT pathway causing STAT1 and STAT3 activation (Heinrich et al., 1998). STAT1 and STAT3 translocate to the nucleus in the form of homo- or heterodimers and bind to the G4 structure within the PD-L1 promoter, inducing PD-L1 gene expression, which will further result in increased protein and surface levels. Interestingly, several DEGs associated with DC activation and maturation are STAT1 or STAT3 targets (TFLink database). Therefore, the proposed mechanism underlying G4-mediated PD-L1 regulation involving the binding of STAT1 and STAT3 might be applicable to several genes that have been impacted by G4 stabilisation in DCs, providing a potential explanation of how G4 stabilisation induces transcriptional programming, resulting in phenotypical and functional changes of DCs. STAT3 plays a critical role in FLT3L-driven DC generation (Laouar et al., 2003). However, multiple studies reported that STAT3 suppresses activation and maturation, and rather promotes a tolerogenic function in DCs (Cheng et al., 2003; Lunz et al., 2007; Melillo et al., 2010; Wang et al., 2004; Wölflle et al., 2011). This is in contrast to the proposed model, which indicated that STAT3 might be involved in the positive regulation of co-stimulatory molecule expression and thus DC maturation and activation. Therefore, future experiments need to investigate the impact of STAT3 on co-stimulatory molecule expression under G4 stabilising conditions to verify

the role of STAT3 in G4-mediated transcriptional regulation in DCs. In contrast to STAT3, STAT1 was described to be important for DC maturation, however, it was only shown to be required for the upregulation of CD40, while CD86 and MHC II regulation is STAT1-independent (Jackson et al., 2004). So far, I have only shown that STAT1 can bind the PD-L1 G4 *in vitro*. Hence, further work on investigating the role of STAT1 in G4-mediated PD-L1 and general transcriptional regulation needs to be conducted in the future.

6.4 Selective targeting of G4s in DCs provides a novel immunotherapeutic approach

Several studies described the importance of PD-L1 expressed on immune cells for efficient response to anti-PD-L1 blockade (Antonios et al., 2017; Herbst et al., 2014; Lin et al., 2018; H. Tang et al., 2018). Furthermore, Peng et al. (2020) demonstrated that PD-L1 expressed on DCs rather than other immune cells plays a critical role in limiting T cell responses, as the therapeutic effect of PD-L1 blockade therapy was completely gone in DC-conditional knockout mice. In this thesis, I showed that G4 stabilisation leads to a strong upregulation of PD-L1 in DCs, which is due to a G4 structure in the PD-L1 promoter that upon G4 stabilisation stimulates PD-L1 expression. Hence, selective targeting of a G4 structure in PD-L1 promoter by G4 ligands leading to increased PD-L1 expression on the DC surface could enhance the efficacy of PD-L1 blockade and improve T cell-mediated anti-tumour responses. This hypothesis is supported by a current study, which demonstrated the use of the G4 ligand CX-5461 in combination with two checkpoint inhibitors on the therapeutic efficacy *in vivo* by increasing PD-L1 expression in CRC (Chung et al., 2023).

So far, multiple G4 ligands have been tested in preclinical evaluation and early phases of clinical trials. However, none of them has been approved yet. The limited success of G4 ligands is mainly due to poor selectivity, low effectiveness and side- and/or off-target effects due to systemic administration (Manaia and Chiorcea-Paquim, 2023; Rizzo et al., 2014; Taetz et al., 2006). Hence, selective

targeting of G4 structures by G4 ligands conjugated with sequence-specific oligonucleotides, peptide nucleic acids or an inactive Cas9 protein would reduce the risk of side and off-target effects (Berner et al., 2024; Cadoni et al., 2023; Qin et al., 2024). Proper delivery of G4 ligands to the target cells by nanoparticles would additionally limit the risk of side- and off-target effects. Due to the important role of DCs in inducing adaptive immunity, directed targeting of G4 ligands to DCs *in vivo* provides a potential strategy to improve adaptive immune responses by simultaneously reducing the risk of G4 ligand-induced side effects.

6.5 Concluding remarks

In summary, this thesis explores the underlying mechanisms of G4-mediated cellular changes in DCs. Importantly, by providing insights into how G4 stabilisation affects DC phenotype and function, it contributes to a better understanding of the role of G4s in cellular pathways relevant for DC regulation. These findings highlight the potential of G4 structures to reprogram DCs to adequately respond to specific environmental cues. This is particularly important as DCs play a crucial role in numerous disease settings, including cancer, viral infections and autoimmune diseases. Future experiments focusing on selective G4 targeting in DCs *in vivo* will broaden the knowledge of G4 structures as a therapeutic target to modulate DC functions to improve existing or develop novel treatment strategies for various diseases.

7. References

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