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Modulation of plasmacytoid dendritic cell function: role of immunoreceptors TIM-3 and BDCA-2

Modulace funkce plazmacytoidních dendritických buněk: role immunoreceptorů TIM-3 a BDCA-2

PhD Thesis

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DECLARATION

I hereby certify that I have written this thesis by my own and that I have not used other than the cited sources. This thesis has not been submitted for any other degree or purposes.

Prohlášení:

Prohlašuji, že jsem disertační práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce nebyla použita k získání jiného nebo stejného akademického titulu.

Mgr. Albert Font Haro

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PREFACE

The present dissertation summarizes the work of three major projects in which I have been involved and that have been already published (Aouar et al., 2016; Font-Haro et al., 2018; Janovec et al., 2018). The work published in 2016 was rather introductory, allowing me to master some of the experiments that I would end performing in the other two works, where I share first authorship.

The body of the work is comprised of a necessary introduction, where I build the background over which I expose the experimental work that follows. The core of the introduction is dedicated to the two major actors of the project, namely plasmacytoid dendritic cells and human immunodeficiency virus, but I have also dedicated a chapter to the relationship of hepatitis C virus and plasmacytoid dendritic cells, given that part of my project concerns it also. I tried to make it as concise and essential as possible without forgetting the bunch of discoveries that have made my whole work possible.

After the presentation, interpretation and discussion of the results, I expose the conclusions to which we arrived. I believe that this work sheds light to the complex and still unknown world of plasmacytoid dendritic cell regulation.

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) are key players in the antiviral response as well as in linking innate and adaptive immune response. They express endosomal toll-like receptors 7 and 9, which can detect ssRNA and unmethylated CpG DNA, respectively. Due to the constitutive expression of the transcription factor IRF7, pDCs are able to rapidly produce massive quantities of type I (α, β, ω) and type III (λ1, λ2, λ3, λ4) interferons (IFN-I and IFN-III) as well as proinflammatory cytokines such as IL-1, IL-6 and TNF-α. After maturation, they also function as antigen-presenting cells. Despite intense research, the mechanisms of IFN and proinflammatory cytokines production and regulation are still poorly understood. Using the pDC cell line GEN2.2 and also primary human pDCs, we shed light on the role of kinases MEK and SYK in IFN-I production and regulation. We found that SYK is not only involved in the regulatory receptor (RR)-mediated BCR-like pathway that represents the negative regulation of IFN-I and IFN-III secretion but also in the positive TLR7/9-mediated signal transduction pathway that leads to IFN-I production, representing the immunogenic function. We also found that MEK plays a crucial role in RRs inhibitory pathway. Further research on pharmacological targeting of SYK and MEK could serve to alleviate the symptoms of diseases caused by the dysregulation of IFN-I production, such as systemic lupus erythematosus (SLE), or conversely, to intensify suppression of viral infections, namely during an acute state of infection when the immune system is not activated enough, a typical situation in HCV, HBV or HIV infections.

In parallel, we studied dynamics of the immunomodulatory phenotypic markers (CD4, BDCA-2, HLA-DR, CD32 and TIM-3) in pDCs of a cohort of 21 treatment-naïve HIV-infected patients and during the first 9 months of the antiretroviral treatment (ART). We found that the expression of these markers was significantly disrupted in treatment-naïve HIV-infected patients in comparison to the controls (healthy donors, HDs). After the 9-month follow-up under ART, the immunogenic phenotype of HIV-infected patients was only partially restored. Importantly, we found a correlation between the levels of expression of TIM-3 in pDCs and the level of decrease of HIV-1 RNA in plasma during the first months on ART. This discovery opens the door to consider TIM-3 as a putative biomarker for antiretroviral treatment efficiency in HIV-infected patients.

ABSTRAKT

Plasmacytoidní dendritické buňky (pDC) hrají klíčovou roli v antivirovou odpověd a propojení vrozené a adaptivní imunity. Tyto buňky exprimují endozomální toll-like receptory 7 a 9 detekující ssRNA a DNA postrádající methylaci CpG dinukleotidů. Díky stálé expresi transkripčního faktoru IRF7 jsou pDC schopny rychlé produkce velkého množství interferonů typu I (IFN-I; α, β, ω), typu III (IFN-III; λ1, λ2, λ3, λ4) a prozánětlivých cytokinů jako jsou IL-1, IL-6 a TNF-α. Po dozrání pDC také fungují jako buňky prezentující antigeny. I přes intenzivní výzkum je mechanizmus produkce a regulace IFN a prozánětlivých cytokinů málo prozkoumaný. S použitím buněčné linie GEN2.2 a primárních lidských pDC jsme popsali roli kináz MEK a SYK v produkci a regulaci produkce IFN-I. Zjistili jsme, že SYK se účastní nejen regulace regulačním receptorem (RR) řízené BCR-like dráhy působící negativně na sekreci IFN-I a IFN-III, ale také v pozitivní signalizaci pomocí TLR7/9 signální dráhy vedoucí k produkci IFN-I. Dále jsme zjistili že také MEK hraje důležitou roli v inhibiční RR dráze. Další výzkum pomocí farmakologického cílení kináz SYK a MEK může posloužit ke zmírnění symptomů nemocí jako je systémový lupus erythematous (SLE), kde je narušena regulace produkce IFN-I, nebo může vést k účinnější potlačení virové infekce během akutní fáze, kdy není dostatečně aktivován imunitní systém, což je situace, ke které dochází při infekci HCV, HBV nebo HIV.

Dále jsme studovali dynamiku markerů imunomodulátorového fenotypu (CD4, BDCA-2, HLA-DR, CD32 and TIM-3) u pDC u kohorty 21 pacientů infikovaných HIV před a prvních 9 měsíců během antiretrovirové terapie (ART). Zjistili jsme, že ve srovnání s kohortou zdravých jedinců dochází u neléčených pacientů k významnému narušení exprese těchto markerů. Po 9 měsíců trvající léčbě pomocí ART docházelo u pacientů jen k částečnému obnovení imunogenního fenotypu. Pozorovali jsme korelaci exprese TIM-3 u pDC a úrovní poklesu RNA HIV-1 v plazmě pacientů během prvních měsícu ART. Tento náš objev otevírá možnost využití TIM-3 jako možného biomarkeru pro hodnocení efektivity ART u pacientů infikovaných HIV-1.

ABBREVIATIONS

A
AGM – African green monkey
AIDS – Acquired immunodeficiency syndrome
APC – Antigen-presenting cell
ART – Antiretroviral therapy
AS DC – $AXL^{+}SIGLEC6^{+}$ dendritic cell
AZT – Azidothymidine
B
BAD-LAMP – Brain and DC-associated LAMP-like molecule
Bcl2 – B-cell lymphoma 2 (protein)
BCR – B-cell receptor
BDCA – Blood dendritic cell antigen
BLNK – B-cell linker (protein)
bNAbs – Broadly neutralizing antibodies
BST2 – Bone marrow stromal cell antigen 2
BTK – Bruton´s tyrosine kinase
BTLA – B- and T-lymphocyte attenuator
BPDCN – Blastic pDC neoplasm
C
CA – Capsid
CAR – Chimeric antigen receptor
CCR9 – C-C chemokine receptor 9
Cas9 – CRISPR-associated protein 9
CD – Cluster of differentiation
CD40L – CD40 ligand
CDC – Common dendritic progenitor
cDNA – Complementary DNA
cGAS – Cyclic GMP-AMP synthase
CLEC4C – C-type lectin domain family 4 member C
CNS – Central nervous system
CpG – Cytosin-phosphate-guanosin oligodeoxynucleotide
CRF – Circulating recombinant form
CRISPR – Clustered regularly interspaced short palindromic repeats
CTL/CLEC – C-type lectin
CTLA-4 – Cytotoxic T-lymphocyte antigen 4
CXCL – C-X-C chemokine ligand
CXCR4 – C-X-C chemokine receptor 4
D
DAAs – Direct-acting antivirals
DAP-12 – DNAX activation protein of 12kDa
DART – Dual-affinity retargeting (molecule)
DC – Dendritic cell
DCIR – Dendritic cell immunoreceptor
DENV – Dengue virus
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
dNTP – Deoxynucleotide triphosphate
DR6 – Death receptor 6
E
E2-2 – E protein 2-2
EBV – Eppstein-Barr virus
ECs – Elite controllers
ELISA – Enzyme-linked immunosorbent assay
ERK – Extracellular signal-regulated kinase
F
Fc – Fragment crystallisable region
FcR – Fc receptor
FcεRI – Fc epsilon RI/ high affinity IgE receptor
FcγRII – Receptor II for the Fc region of IgE
FCS – Fetal calf serum
Flt3 – Fms like tyrosine kinase 3
Flt3L – Fms like tyrosine kinase 3 ligand
FMO – Fluorescence minus one (control)
G
GALT – Gut-associated lymphoid tissue
G.eq – Genomic equivalence
GIT – Gastrointestinal tract
GM-CSF – Granulocyte macrophage colony-stimulation factor
GM-CSFR – GM-CSF receptor
Gp – Glycoprotein
H
HAART – Highly active antiretroviral therapy
HAV – Hepatitis A virus
HBV – Hepatitis B virus
HCV – Hepatitis C virus
HCVcc – HCV derived from cell culture
HD – Healthy donors
HDACis – Histone deacetylase inhibitors
HIV – Human immunodeficiency virus
HLA – Human leukocyte antigen
HLA-DR – HLA-DR isotype
HMGB1 – High mobility group box 1
HSCT – Hematopoietic stem cell transplantation
I
IBD – Inflammatory bowel disease
IC_{50} – Half maximal inhibitory concentration
ICOS-L – Inducible T-cell costimulator ligand
ID2 – Inhibitor of DNA binding 2
IDO – Indoleamine 2,3-dyoxigenase
IFN – Interferon
IFNAR – IFN alpha receptor
IKK-α – Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKpDC – IFN-producer Killer pDC
IL – Interleukin
ILT – Immunoglobulin-like transcript
IN – Integrase (viral)
IQR – Interquartile range
IRAK – Interleukin-1 receptor-associated kinase
IRF7 – Interferon regulatory factor 7
IRIS – Immune reconstitution inflammatory syndrome
ISG – Interferon stimulated genes
ITAM – Immunoreceptor tyrosine-based activation motif
ITIM – Immunoreceptor tyrosine-based inhibitory motif
J
JNK – c-Jun N-terminal kinase
K
Kdr – Kinase insert domain receptor (gene)
L
LAMP – Lysosome-associated membrane glycoprotein
LCMV – Lymphocytic choriomeningitis mammarenavirus
LFA-1 – Lymphocyte function-associated 1
LN – Lymph node

LRA – Latency-reversing agents
LTR – Long terminal repeat

M

MA – Matrix (viral)
mAb – Monoclonal antibody
MAPK – Mitogen-activated protein kinase
MAPKKK – MAP kinase kinase kinase
MAP3K7IP2 – MAP kinase kinase kinase 7-interacting protein 2
MARCH1 – Membrane-associated RING-CH finger protein 1
M-CSFR – Macrophage-colony stimulation factor receptor
mDC – Myeloid dendritic cell
MEK – MAPK-ERK-kinase
MEKi – MEK inhibitor
MEM – Minimal essential medium
MHC – Major histocompatibility complex
miR – Micro RNA
MOI – Multiplicity of infection
m-TOR – Mammalian target of rapaMycin
MyD88 – Myeloid differentiation primary response 88

N

NCR-2; Nkp44 – Natural cytotoxicity triggering receptor 2
Nef – Negative regulatory factor
NET – Neutrophil extracellular trap
NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT – Nuclear factor of activated T-cells
NK – Natural killer
NNRTI – Non-nucleoside reverse transcriptase inhibitor
NOD – Non-obese diabetic (mouse model)
Nkp44 – See NCR-2
NRTI – Nucleoside reverse transcriptase inhibitor

O

ODN – Oligodeoxynucleotide
OPN – Osteopontin
ORF – Open reading frame
OX40L/TNFRSF4L – TNF receptor superfamily 4 ligand

P

PBMC – Peripheral blood mononuclear cells
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
PD1 – Programmed death protein
pDC – Plasmacytoid dendritic cell
PD-L1 – Programmed death-ligand 1
pET – PDC extracellular trap
PI3K – Phosphoinositide-3-kinase
PIC – Pre-integration complex
PMA – Phorbol 12-myristate 13-acetate
PTCs – Post-treatment controllers
PT – Protease (viral)
PTPRS – Receptor-type tyrosine-protein phosphatase S

Q

QVOA – Quantitative viral outgrowth assay

R

Rev – Regulator of expression of virion proteins
RIG-I – Retinoic acid inducible gene I
RING – Really interesting new gene
RM – Rhesus macaque
RNA – Ribonucleic acid
RNAi – Interference RNA
RNApolII – RNA polymerase type II
RPMI – Roswell park memorial institute (cell culture medium)
RR – Regulatory receptor
RT – Reverse transcriptase

S

SAMHD1 – Sterile alpha motif (SAM) domain and histidine-aspartic (HD) domain-containing protein 1
SEM – Standard error of the mean
SHIV – SIV-containing HIV envelope
SIGLEC – Sialic acid-binding Ig-like lectin
SIV – Simian immunodeficiency virus
SIVcpz*ptt* – SIV chimpanzee (*pan troglodytes troglodytes*)
SIVgor – SIV gorilla
SIVsmm – SIV sooty mangabey
SLE – Systemic lupus erythematosus
SM – Sooty mangabey
SSc – Systemic sclerosis
ssRNA – Single stranded RNA
STAT3 – signal transducer and activator of transcription 3
STING – Stimulator of IFN genes
SU – Surface protein (viral)
Syk – Spleen tyrosine kinase

T

T/F – Transmitter/Founder
TAK1 – Transforming growth factor β-activated kinase 1
TAB2 – TGF-beta-activated kinase 1 and MAP3K7-binding protein 2
TApDC – Tumor-associated pDC
Tat – Trans-activator of transcription
TBK1 – TANK binding kinase 1
TCR – T-cell receptor
TGF – Transforming growth factor
Th17 – T-helper 17 lymphocytes
TILDA – Tat/rev induced limiting dilution assay
TIM-3 – T-cell immunoglobulin and mucin domain-containing 3
TM – Transmembrane region
TNF-α – Tumor necrosis factor α
TNFRSF21 – Tumor necrosis factor receptor superfamily 21
TLR – Toll-like receptor
TRAF – TNF receptor-associated factor
TRAIL – TNF-related apoptosis-inducing ligand
T_{reg} – T regulatory lymphocyte

U

UNC93B1 – Unc-93 homolog B1

W

WNV – West nile virus

V

VEGFR2 – Vascular endothelial growth factor receptor 2
Vif – Viral infectivity factor
VL – Viral load
Vpr – Viral protein R
Vpu – Viral protein U

Vpx – Viral protein X
VR – Viral Rebound
VSV – Vesicular stomatitis virus
Y
YFV – Yellow fever virus
Z
ZDV – Zidovudine

CONTENTS

1. HYPOTHESES AND AIMS

a) Evaluate, characterize and compare the immunological profile focusing on plasmacytoid dendritic cells of the 21 HIV-infected patients and their matched controls before and after the onset of antiretroviral therapy at different time points (0, 3, 6 and 9 month after the onset of antiretroviral therapy). The characterization of the immunological profile is done by flow cytometric analysis. Then, focus in the search of HIV infection-derived exhaustion surface markers in plasmacytoid dendritic cell. Specially, TIM-3, since it has been described as an exhaustion marker of plasmacytoid dendritic cells during HIV-1 infection, but only at a given time point. We hypothesise that TIM-3 may be a predictor and an indicator of infection progression and immunological state during HIV-1 infection.

b) To evaluate the role of the tyrosine kinase SIK in the regulation of toll-like receptor signalling and interferon production as well as proinflammatory cytokine production in plasmacytoid dendritic cells. We hypothesize that SIK may be a keystone in the regulation of interferon and cytokine production in plasmacytoid dendritic cells and therefore an attractive target for immunomodulatory drugs.

c) To evaluate the role of the MAP kinases MEK and ERK in the regulation of toll-like receptor signalling and interferon and proinflammatory cytokines production in plasmacytoid dendritic cells. We hypothesize that MEK and ERK may play important roles in the regulation of interferon and cytokine production in plasmacytoid dendritic cells and therefore an attractive target for immunomodulatory drugs.

2. INTRODUCTION

2.1. Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDCs) are a highly specific dendritic cell subset that have an important role in linking innate and adaptive immunity. They have a potent antiviral and antitumor activity and can also be involved in autoimmune diseases (Ah Kioon et al., 2018; Alculumbre et al., 2019, 2018; Brewitz et al., 2017; Reizis, 2019; Swiecki & Colonna, 2015). PDCs express endosomal Toll-like receptors 7 and 9 (TLR7/9) with which they can detect single-stranded RNA (ssRNA) and unmethylated CpG DNA respectively. After TLR7/9 challenge with its ligands, pDCs trigger a signalling pathway that ends with the massive production of type I (α, β, ω) and type III (λ1, λ2, λ3, λ4) interferons (IFN-I and IFN III), as well as other proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α), finally maturing into a fully functional antigen-presenting cell (APC) with the ability of priming $CD4^+$ T cells and cross-present antigen to $CD8^+$ T cells. The rapid production of IFN I/III by pDCs induces the expression of interferon-stimulated genes (ISGs) at a paracrine and autocrine level and allows the establishment of an antiviral state in the neighbouring cells. (Bao & Liu, 2013; Gilliet, Cao, & Liu, 2008; Hirsch, Caux, Hasan, Bendriss-Vermare, & Olive, 2010; Hirsch, Janovec, Stranska, & Bendriss-Vermare, 2017; Leifer & Medvedev, 2016; Swiecki & Colonna, 2015) **(Figure 1)**.

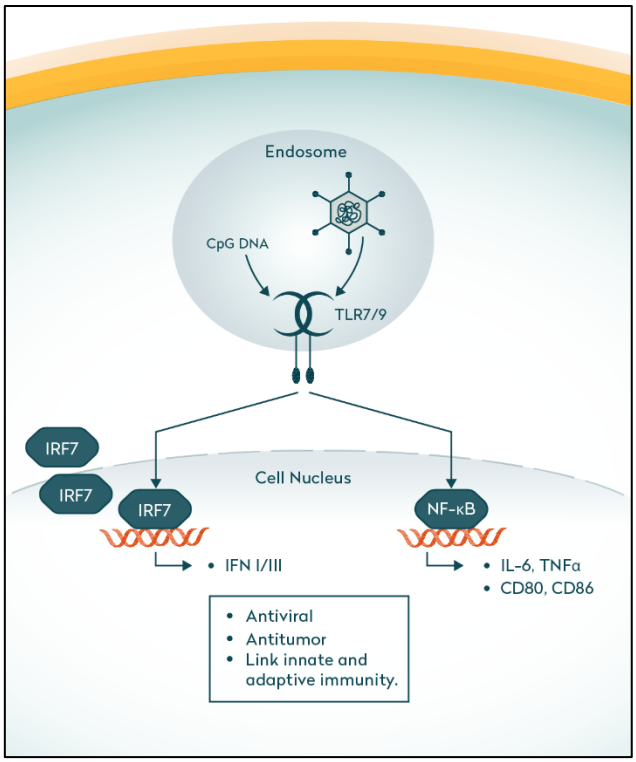

Figure 1: Main functions of plasmacytoid dendritic cells. Plasmacytoid dendritic cells are able to sense viral nucleic acids or synthetic CpG-ODNs by means of the endosomal receptors TLR7 and TLR9. TLR7 binds ssRNA or synthetic imidazoquinoline compounds (e.g. R848) and TLR9 binds viral and bacterial DNA as well as synthetic CpG oligodeoxynucleotides (e.g. CpG-A; CpG-B). Depending on the stimulus either an IRF7-dependent or an NF-κB-dependent genic induction takes place, leading the cell either to become a professional IFN-I/III producer or to secrete proinflammatory cytokines (e.g. IL-6, TNF-α) and develop antigen-presenting features with the upregulation of costimulatory molecules (e.g. CD80, CD86) and MHCII. PDCs participate on antiviral as well as antitumoral immunity. They have been proven to have a role in some autoimmune diseases such as SLE or Psoriasis.

CpG-ODN, CpG-oligodeoxynucleotides; TLR7/9, toll-like receptor; IRF7, interferon regulatory factor 7 ; NF-κB, nuclear factor-κB; IFN, interferon; IL, interleukin; APC, antigen-presenting cell; MHCII, major histocompatibility complex II; TNF-α, tumor nuclear factor α; SLE, systemic lupus erythematosus.

2.1.1. Ontogeny and phenotype

pDCs can develop from either lymphoid or myeloid precursors, however, experiments in mice showed that favoring the depletion of lymphoid progenitors via estrogen treatment does not affect pDC numbers, indicating that non-lymphoid precursors play the major role in pDC production (Alculumbre et al., 2018; Harman, Miller, Nikbakht, Gerstein, & Allman, 2006;

Swiecki & Colonna, 2015). A common dendritic cell (DC) progenitor (CDC) is the precursor of both myeloid dendritic cells (mDCs) and pDCs and is characterized by the expression of Fms-like tyrosine kinase 3 (Flt3; CD135), macrophage colony-stimulating factor receptor (M-CSFR; CD115) and a mild expression of the receptor tyrosine kinase KIT (CD117) (Naik et al., 2007; Onai et al., 2007; Satpathy, Wu, Albring, & Murphy, 2012). Flt3 engagement with its ligand Flt3L is essential for the development and expansion of the DC progenitors. Flt3 signalling leads to the activation of the signal transducer and activator of transcription 3 (STAT3) and phosphoinositide 3-kinase (PI3K)-dependent activation of mammalian target of rapamycin (m-TOR) (Laouar, Welte, Fu, & Flavell, 2003; Sathaliyawala et al., 2010). Importantly, experiments in mice showed that pDCs are more dependent on Flt3 stimulation for its development than mDCs given that if Flt3 is absent pDCs are selectively depleted from bone marrow and lymphoid organs (Eidenschenk et al., 2010; Waskow et al., 2008). The transcription factor E2-2 (also known as TCF4), a basic hemophagocytic lymphohistiocytosis protein, drives the commitment of the CDP to become pDC and is antagonized by the transcription factor ID2, an inhibitor of DNA binding, which if active, leads to the development of mDCs from CDP in detriment of pDCs. Thus, the balance between E2-2 and ID2 is an important factor in determining the fate of the CDPs. ID2 is almost absent in CDP and pDCs but abundantly expressed in mDCs. To sum up, a continuous expression of E2-2 and low levels of ID2 are required to maintain pDCs identity and avoid the spontaneous differentiation into mDC-like cells (Murphy et al., 2016; Spits, Couwenberg, Bakker, Weijer, & Uittenbogaart, 2000; Tussiwand & Gautier, 2015).

The phenotypic markers present on pDCs are CD123, CD45RA, CD4 and the regulatory receptors (RRs) blood dendritic cell antigen 2 (BDCA-2; CD303; CLEC4C), BDCA-4 (CD304; neurophilin-1), immunoglobulin-like transcript 3 (ILT-3; CD85k), ILT-7 (CD85g), high-affinity IgE receptor 1 (FcεR1), B- and T-lymphocyte attenuator (BTLA), death receptor 6 (DR6; TNFRSF21; CD358) and CD300A. Unlike mDCs, pDCs do not express the myeloid antigens CD11c, CD33, CD11b or CD13 (Bao & Liu, 2013; Dzionek et al., 2000, 2001; Ju, Zenke, Hart, & Clark, 2008; MacDonald et al., 2002). CD4 is expressed in both lineages but at a higher level in pDCs (L. Jardine et al., 2013).

Since the characterization of pDC as a new cell population, experiments with human pDCs have been difficult to carry on because of the low numbers of these cells in circulation. Cutting edge technologies have allowed the definition of pDCs subsets characterized by different marker expression and function. The big question in the field is whether the functionally distinct pDC subsets are settled early during the developmental stage, or if the pDC pool has the capacity to diversify *in situ* in either direction depending on the environmental signals **(Figure 2)**.

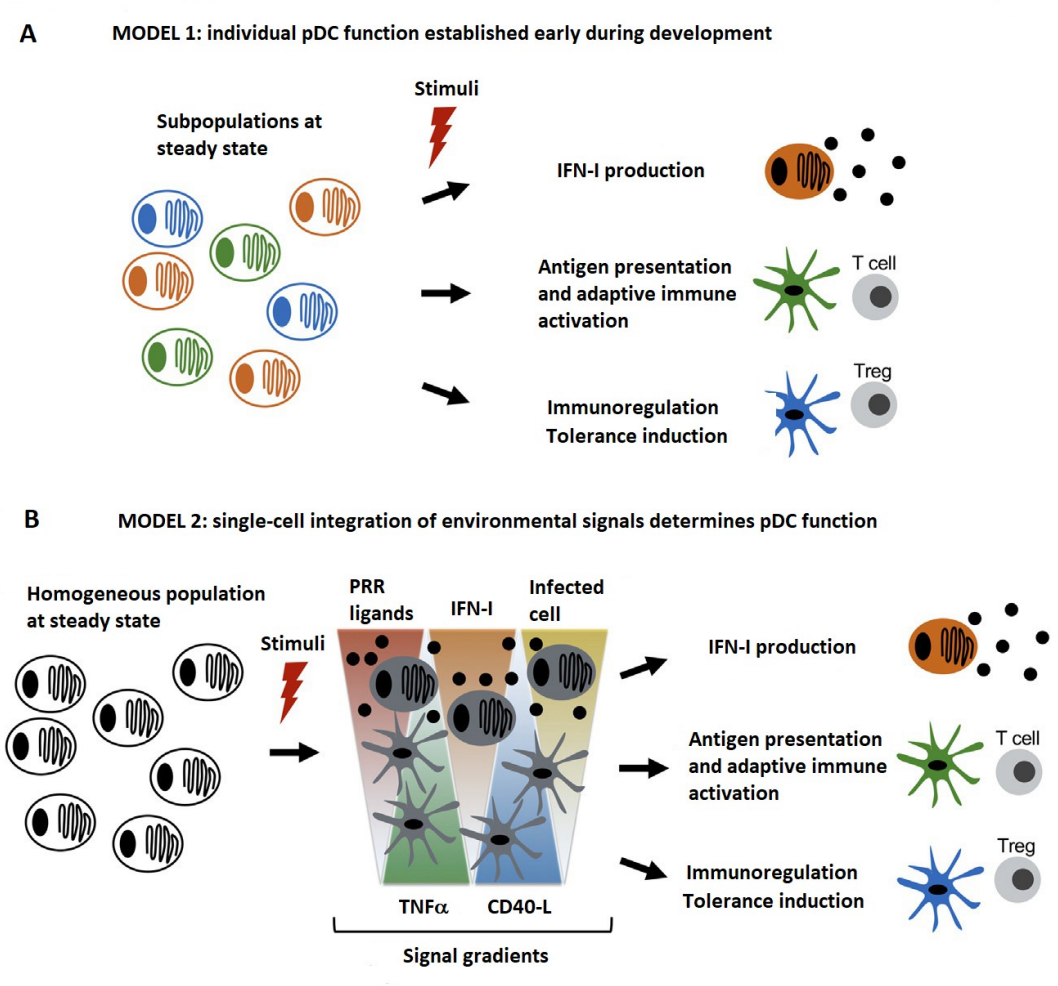

Figure 2: Models of pDC functional diversity. (A) In the model 1 the functions acquired after pDC activation are pre-established during development. The division of labour among the pDC pool will depend on it. (B) In the model 2 pDC will respond individually to the environmental stimulus and acquire its function depending on the intensity and nature of the stimuli. Neighbouring cells integrate the signals that ultimately will lead to functional heterogeneity among the pDC pool. *Adapted from Leylek & Idoyaga, 2019.*

PRR, pattern-recognition receptor; Treg, T regulatory cell.

Following the hypothesis that presumes that pDC functional heterogeneity is developmentally encoded, studies have shown small alternative pDCs subpopulations defined by the expression of CD2 (Bryant et al., 2016; Matsui et al., 2009), CD56 (Osaki et al., 2013; H. Yu et al., 2015) or CD5 (H. Zhang et al., 2017) in which, interestingly, distinctive gene expression patterns overlap with conventional mDCs. Similarly, single-cell RNA sequencing (RNA-Seq) and mass cytometry (CyTOF) revealed a rare circulating DC population which presents a pDC-like phenotype (Alcántara-Hernández et al., 2017; See et al., 2017; Villani et al., 2017). This group

displays common pDC markers such as CD123, BDCA2 or BDCA4, but, unlike canonical pDCs, also express the mDC markers CD33 and CD11c, resulting in an intermediate phenotype between pDCs and mDCs. This subpopulation express also AXL receptor tyrosine kinase and sialic acid-binding Ig-like lectin 1 (SIGLEC1/6) and have been defined as $AXL^{+}SIGLEC6^{+}$ DC (AS DC) or DC5 (Villani et al., 2017). Far from being a discrete population, high dimensional analysis at the protein level of samples from several tissues, revealed that these cells show a continuum of heterogeneous phenotypes ranging from pDC-like to mDC-like (Alcántara-Hernández et al., 2017). These features were also reported by single-cell RNA-Seq analysis (Villani et al., 2017). Interestingly, the pDC-defining transcription factor E2-2 was found to be expressed at varying levels across the population correlating with how pDC-like or mDC-like the phenotype showed (Alcántara-Hernández et al., 2017). AS DCs don´t have the capacity of producing great quantities of IFN-I after TLR stimulation but they were shown to have a more potent capacity of inducing T cell activation similar to that of mDCs. However, it is still unclear if these cells have a unique function that distinguishes from pDCs and mDCs (See et al., 2017; Villani et al., 2017).

Regarding the second hypothesis, which states that functional diversity of pDCs is not predisposed during the development but emerges after stimulation, one of the most striking characteristics of pDCs is the ability to undergo a cell state conversion or transdifferenciation and become an mDC-like antigen-presenting cell *in vitro*. This evidence of functional plasticity, in which, a differentiated cell is able to alter its phenotype and function in response to environmental stimuli has also been shown in macrophages, mast cells, and neutrophils (Galli, Borregaard, & Wynn, 2011). A study in which pDCs where purified by excluding the newly described DC AS subset showed that pDCs maintain the ability to become mDC-like antigen-presenting cells and induce T cell proliferation after the exposure to IL-3 + CD40L or virus (Alcántara-Hernández et al., 2017; Alculumbre et al., 2019, 2018). It is important to note that in this way, the antigen-presentation capacity of pDCs cannot be attributed to DC AS contamination, suggesting that pDCs are in fact exceptionally plastic. Alculumbre et al., Defined three different pDC subpopulations depending on the levels of expression of the surface markers programed death-ligand 1 (PD-L1) and CD80 after stimulation. These subpopulations perform different specialized functions in the range of innate immunity (Alculumbre et al., 2018). Thus, P1-pDCs ($PD\text{-}L1^{+}\ CD80^{-}$) present a plasmacytoid morphology, which is defined by abundant endoplasmic reticulum and a plasmatic cell look, and are specialized in the production of IFN-I. On the other hand, P3-pDCs ($PD\text{-}L1^{-}CD80^{+}$) present dendritic morphology and are specialized in antigen presentation and proinflammatory cytokines production. A third subset, P2-pDCs ($PD\text{-}L1^{+}\ CD80^{+}$) performs both adaptive and innate functions. Each one of the subsets show a stable

and specific transcriptomic signature (Alculumbre et al., 2018). Importantly, the mechanisms that promote this functional diversity are not known. The evidence exposed strengthen the idea that slight variations in the signals received and integrated by single cells are responsible for functional diversification of pDCs (Leylek & Idoyaga, 2019). The process of diversification *in situ* could be advantageous in disease, giving to the cell the possibility to adapt to concrete circumstances or infectious agents, in order to respond accordingly to the specific threat. In addition to the aforementioned distinct immunogenic phenotypes of pDCs in response to concrete stimuli, it would be advantageous to study if and to which degree are the different pDC subsets capable to adapt to situations in which immunotolerance is necessary (e.g. autoimmune diseases in which pDCs have been proven to be involved, such as systemic lupus erythematosus (SLE) or systemic sclerosis (Eloranta et al., 2010; D. Kim et al., 2008; Panda, Kolbeck, & Sanjuan, 2017), given the ability of pDCs to induce the generation of T regulatory (Treg) cells (Hanabuchi et al., 2010; Ito et al., 2007; Moseman et al., 2004).

2.1.2. PDC activation and its mechanisms

The activation of pDCs is essential in the context of a viral infection because of the importance of a rapid and strong IFN-I response early during the onset of infection and also because of the role they play in linking innate and adaptive immunity (Lande & Gilliet, 2010). The canonical model describes two main ways in which a pDC can evolve depending on the specific signal that triggers its activation. On one hand, it can become a professional interferon-producing cell, a potent producer of type-I-interferon. On the other hand it can become a producer of proinflammatory cytokines and mature into an APC with the ability of priming T cell-mediated adaptive immune responses (Grouard et al., 1997; Swiecki & Colonna, 2015).

The current view on pDC activation is based in the spatiotemporal hypothesis (Honda et al., 2005). The results published by Honda et al., suggested that TLR9-dependent pDC activation in either IRF7-directed professional IFN-1 producer or nuclear factor-κB (NF-κB)-directed producer of proinflammatory cytokines and later fully mature APC depends in part on the subcellular localization of TLR9 engagement with the corresponding pathogen-associated molecular pattern (PAMP). Later publications by others detailed the molecular and cellular mechanisms of these events. First of all, the membrane protein UNC93B interacts with TLR9 and transports it from the endoplasmic reticulum to the endosomal compartment (Brinkmann et al., 2007; Y.-M. Kim, Brinkmann, Paquet, & Ploegh, 2008) **(Figure 3)**. It is important to remark that either for the IRF7

pathway as well as for the NF-κB pathway, the previous trafficking of TLR9 is mandatory and is ruled by UNC93B. Another requirement for TLR9 to signal is the cleavage of its ectodomain, carried out by cathepsins in the endolysosomes (Ewald et al., 2008; Park et al., 2008). It has been shown that the retention of synthetic CpG-A oligodeoxynucleotides (ODNs) in early endosomes is associated with the massive production of IFN-I whereas the rapid mobilization of CpG-B ODNs to late endosomes is associated with NF-κB activation (Guiducci et al., 2006; Honda et al., 2005). From this point on, several studies regarding the sorting step identified essential actors. Sasai et al., demonstrated that pDCs isolated from mice that are deficient in adaptor protein-3 (AP-3), a protein complex that has been shown to recruit cargo in endosomes and deliver it to lysosome-related organelles, are deficient in late endosomal transport and IFN-I production but not deficient in NF-κB-dependent proinflammatory cytokines production (Sasai, Linehan, & Iwasaki, 2010), therefore, identifying AP-3 as an essential molecule for IFN-I production. However, a more recent *in vivo* study in mice with natural TLR viral ligands questioned this model. Tomasello et al., showed that during mouse cytomegalovirus infection, neither the participation of AP-3, nor high basal levels of IRF7, nor IFN-I feedback were necessary for a robust IFN-I response (Tomasello et al., 2018).

The brain and DC-associated lysosome-associated membrane glycoprotein (BAD-LAMP/LAMP5) molecule, which is expressed in the nervous tissue of most metazoan species, is also expressed in non-activated human pDCs as well as in blastic pDC neoplasms (BPDCN) from leukemic patients. BAD-LAMP has been shown to control the sorting of TLR9 in different endosome subsets and to favor NF-κB-dependant proinflammatory cytokine production in the BPDCN-derived pDC cell line CAL-1 as well as in primary pDCs (Combes et al., 2017). Upon CpG activation BAD-LAMP is transported along with TLR9 to IRF7-promoting early endosomes, and subsequently to $LAMP1^{+}$ NF-κB-promoting late endosomes. Interestingly, inhibition of BAD-LAMP leads to the retention of TLR9 in early endosomes and increased IFN-I expression. Using confocal microscopy, early endosomes have been shown to contain the SNARE protein vesicle-associated membrane protein3 (VAMP3) and LAMP2, but not LAMP1. In the same study they also have shown that exposure to immunosuppressive cytokines or tumor supernatants prevent down-modulation of BAD-LAMP, which occurs rapidly after CpG activation in normal conditions, leading to the polarization towards an NF-κB-directed pro-inflammatory cytokine producer phenotype, and as a consequence IFN-I production is limited (Combes et al., 2017).

In recent years, several investigations refined the model of the molecular mechanisms that lead to pDC activation. As mentioned above, the major function of pDCs is the sensing of viral

nucleic acids via endosomal TLRs: TLR7 recognizes ssRNA and also recognizes synthetic imidazoquinoline species, such as resiquimod (R848), whereas TLR9 recognizes viral DNA as well as synthetic CpG oligonucleotides. Engagement of aggregating CpG-A ODN in the early endosome unchains an AP3-dependent myeloid differentiation primary response 88 (MyD88)-IRF7 pathway that involves the adaptor protein MyD88, interleukin-1 receptor-associated kinase 1/4 (IRAK 1/4), TNF receptor-associated factors 3 and 6 (TRAF3/6), and ultimately IRF7. IRF7 translocates to the nucleus and together with NF-κB subunits p50 and RelA, and the transcription factors ATF-2 and c-Jun, which conform AP-1, initiates the production of IFN-I and III (Bao & Liu, 2013; Gough, Messina, Clarke, Johnstone, & Levy, 2012; Leifer & Medvedev, 2016; Sasai et al., 2010; Swiecki & Colonna, 2015). On the other hand, ligation of TLR9 with monomeric CpG-B oligonucleotides develops in the transfer to an endolysosomal compartment where the pathway MyD88-NF-κB is activated, inducing the formation of a signalosome composed by IRAK2/4 and TRAF6 which activates transforming growth factor β-activated kinase 1 (TAK1) and subsequently the mitogen-activated protein kinases (MAPKs) and the transcription factor IRF5 (Purtha, Swiecki, Colonna, Diamond, & Bhattacharya, 2012; Takaoka et al., 2005) which along with NF-κB and AP-1 unchain the production of the proinflammatory cytokines IL-6 and TNF-α, several chemokines and the expression of the co-stimulatory molecules CD80 and CD86. Importantly, IRAK2 suppresses the production of IFN I/III but induces the production of proinflammatory cytokines (Bao & Liu, 2013; Röck et al., 2007; Swiecki & Colonna, 2015) **(Figure 3)**.

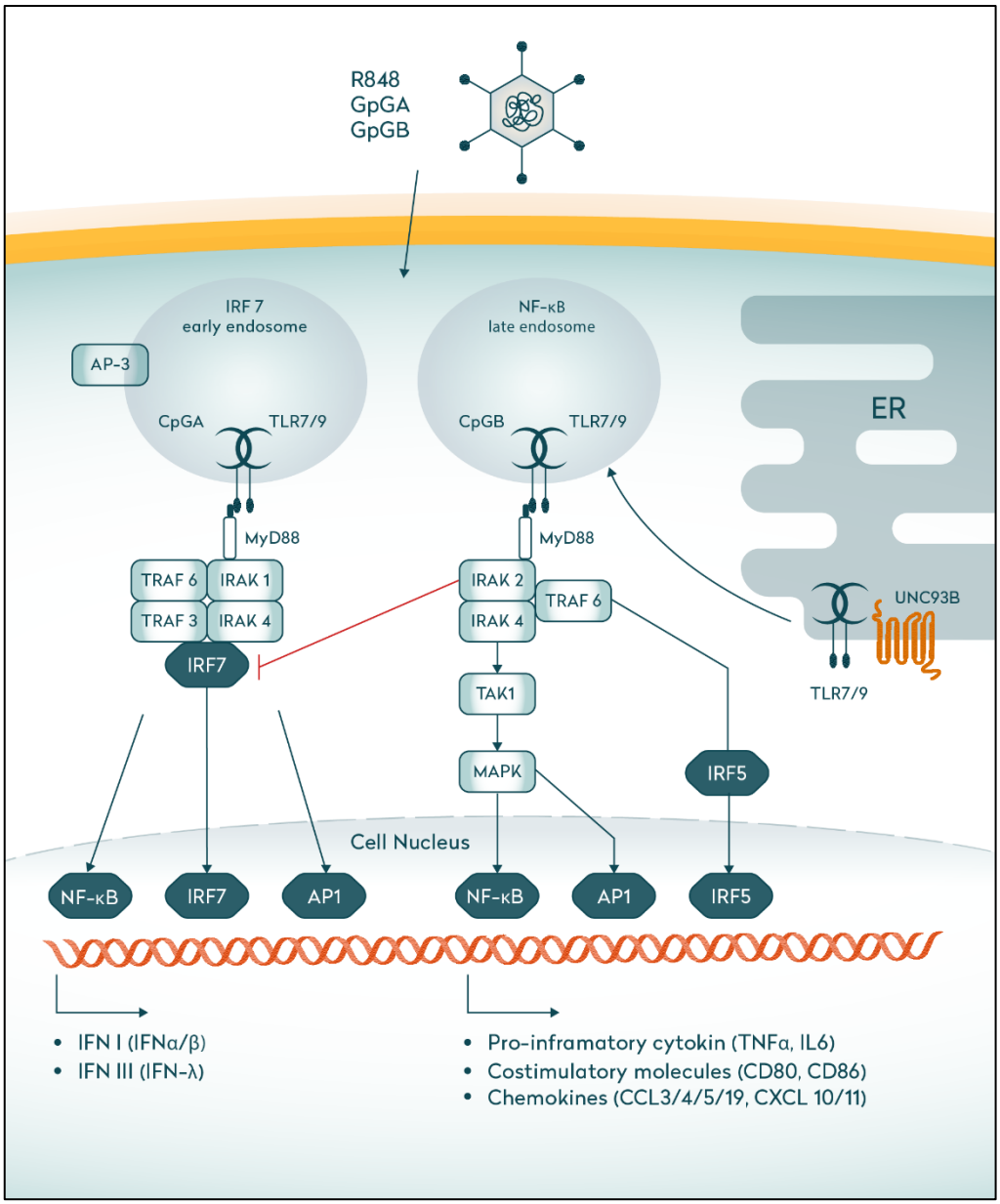

Figure 3: TLR7/9 signalling after ligation in pDCs. TLR7/9 is transported from the endoplasmic reticulum to the endosomes complexed with the transmembrane protein UNC93B. Ligation of the endosomal TLR7/9 with the synthetic agonists CpG-A or CpG-B induces different signalling pathways in pDCs. CpG-A-AP3-dependent signalling from early/IRF7 endosomes through the adaptor protein MyD88 triggers the formation of a signalosome composed of TRAF6, TRAF3, IRAK1 and IRAK2. This signalosome activates IRF7 along with NF-κB and AP-1 which promotes the production of IFNs I (IFN-α/β) and III (IFN-λ). On the other hand, ligation with CpG-B triggers an also MyD88-dependant signalling pathway in late/ NF-κB endosomes. After MyD88 is activated a signalosome composed of IRAK2, IRAK4 and TRAF6 is engaged. The signalosome activates TAK1, which in its turn activates MAPKs leading to the translocation to the nucleus of the transcription factors NF-κB, AP-1 and IRF5. Ultimately these events lead to the induction of proinflammatory cytokines (TNF-α, IL-6), chemokines (CCL3/4/5/19, CXC10/11) and

costimulatory molecules (CD80, CD86). IRAK2 downmodulates IFN-I/III production but promotes proinflammatory cytokines secretion. *Adapted from Hirsch et al., 2017.*

TLR, toll-like receptor; ER, endoplasmic reticulum; UNC93B, Unc-93 homolog B; AP-3, adaptor protein-3; MyD88, myeloid differentiation primary response 88; TRAF, TNF receptor-associated factor; IRAK, interleukin-1 receptor-associated kinase; TAK1, transforming growth factor β-activated kinase 1; MAPK, mitogen-activated protein kinase; IRF, interferon regulatory factor; NF-κB, nuclear factor- κB; AP-1, activator protein 1; IFN, interferon; TNF-α, tumor necrosis factor-α; IL, interleukin; CCL, chemokine (C-C motif) ligand; chemokine (C-X-C motif) ligand, CXCL.

2.1.3. Production of Interferons and proinflammatory cytokines by pDCs

Interestingly, within 6h of viral-induced activation, 60% of pDC transcriptome is due to IFN-I genes (Ito, Kanzler, Duramad, Cao, & Liu, 2006). The ability to produce massive amounts of IFN-I relatively fast can be explained because in contrast to most cell types, pDCs express constitutively high levels of the transcription factor IRF7, which is the main factor involved in the transcription of IFN I genes. On the other hand, IRF7 levels are low in any other cell type and the production of IFN-I depends exclusively on a positive feedback that depends on the previous secretion IFN-β (a subtype of IFN-I that acts at an autocrine level) and the IFN-I receptor IFNAR. Then, engagement of IFNAR with IFN-β leads to the upregulation of ISGs, among which we find IRF7, and the subsequent production of IFN-α in significant quantities. Significantly, while pDCs can produce IFN-I directly after TLR sensing and independently of IFNAR-based feedback signalling, most cell types require this feedback loop that delays considerably the IFN-I production (Barchet et al., 2002; Dalod et al., 2002).

A non-endosomal signalling pathway working also through the adaptor MyD88 may be triggered after the sensing of certain stimuli by TLR2 (Dasgupta, Erturk-Hasdemir, Ochoa-Reparaz, Reinecker, & Kasper, 2014). Regarding intracellular sensors which lead to the production of IFN-I, cyclic GMP-AMP synthase/stimulator of IFN genes (cGAS/STING) and retinoic acid-inducible gene I (RIG-I) may be functional in pDCs in response to DNA (X.-D. Li et al., 2013) and RNA (Bruni et al., 2015; Kumagai et al., 2009) of viral origin, respectively, but the role of the aforementioned intracellular receptors is thought to be residual when compared to endosomal TLR7/9.

Apart from type-I-IFNs and other proinflammatory cytokines, pDCs are able to produce also significant quantities of type-III (especially IFN-λ3) in response to certain stimuli. Importantly, peripheral blood pDCs have been found to be the main IFN-λ3 producer (O'Connor et al., 2014; Stone et al., 2013). IFN-III production by pDCs is JAK/STAT and IRF7-dependent in a similar way that IFN. Recent investigations found the production of IFN-III by pDCs seems to play a role in

the onset of systemic lupus erythematosus (Gilliet et al., 2008; Hjorton, Hagberg, Pucholt, Eloranta, & Rönnblom, 2020; V. C. Lombardi & Khaiboullina, 2014). Further studies in this sense are necessary. Importantly, pDC-derived IFN-λ3 has been shown to be a key factor for hepatitis C virus (HCV) clearance (Ge et al., 2009; Thomas et al., 2009).

2.1.4. PDCs as antigen presenting cells

The major function of pDCs is the quick production of type-I-IFN, however, given that they express MHC class II and the costimulatory molecules CD40, CD80 and CD86, they can also efficiently prime and cross-prime T cells (reviewed in Reizis, Bunin, Ghosh, Lewis, & Sisirak, 2011; Swiecki & Colonna, 2015; Villadangos & Young, 2008). pDCs have been often attributed a weak antigen-presenting capacity but recent studies showed that their abilities in priming T cells may overlap with the ones of mDCs. Thus, the difference on the function of these two subsets DCs may be qualitative rather than quantitative. pDCs require TLR-mediated activation to be able to prime T cells (Mouries et al., 2008; Young et al., 2008). A specific antigen-presentation pathway that operates in pDCs but not mDCs is the proteasome-independent endosomal pathway of viral antigen cross-presentation to cytotoxic $CD8^+$ T cells. This particular mechanism of pDCs is possible thanks to the recycling endosomes in which peptides are continuously loaded to MHC class I molecules (Di Pucchio et al., 2008). Another difference of pDCs compared to mDCs antigen presentation is that pDCs continuously synthesize MHC class II after activation, also to facilitate the presentation of viral peptides (Sadaka, Marloie-Provost, Soumelis, & Benaroch, 2009; Young et al., 2008). Depending on the context, antigen presentation by pDCs can lead to $CD4^+$ T cell activation or to tolerance induction. If pDCs receive stimulus through TLRs, the output will be immunogenic. On the other hand, if they either remain unstimulated or are alternatively activated leading to the expression of indoleamine 2,3-dyoxigenase (IDO) (Boasso et al., 2007; Fallarino et al., 2004; Munn et al., 2004; Pallotta et al., 2011), inducible T cell costimulator ligand (ICOS-L; CD275) (Ito et al., 2007), tumor necrosis factor ligand superfamily member 4 (TNFSF4;OX40L) (Diana et al., 2009), PD-L1 (Diana et al., 2011), or granzyme B (Jahrsdorfer et al., 2010), the result will be tolerance induction. Tolerance induction can be directed towards harmless antigens, tumor cells, or alloantigens. Studies reported that $CCR9^+$ pDCs, which acquire antigenic cargo in peripheral tissues, use CCR9 to migrate to the thymus, where they can induce the depletion of antigen-specific thymocytes and contribute in this way to central immune tolerance (Hadeiba et al., 2012). In a similar way, pDCs can migrate to lymph nodes to promote tolerance (Kohli, Janssen, & Forster, 2016). As pDCs are potent inducers of T_{reg}, studies have

implicated them in being responsible of the inhibition of acute graft-versus-host disease provoked by allogeneic $CD4^+$ donor T cells in irradiated recipients (Hadeiba et al., 2012). Contrarily, they have been recently proved as important players in human kidney allograft rejection (Reich, Viehmann, & Kurts, 2018). To get deeper knowledge about the antigen presentation capacity of pDCs, antigens have been targeted to pDCs surface molecules (especially to regulatory receptors (RRs)) by conjugating them to specific antibodies. Thus, a study showed that antigens targeted to the C-type lectin receptor BDCA2 promotes tolerance by suppressing antigen-specific $CD4^+$ T cell and antibody responses upon secondary exposure to antigen in the presence of adjuvant (Chappell et al., 2014). Similarly, antigens directed to the RR DC immunoreceptor (DCIR) not only inhibit TLR9-mediated IFN-α production but also the antigen is efficiently presented to T cells. Antigen presentation to T cells by DCIR promote T cell proliferation. The report does not specify if the T cell proliferation leads to an immunogenic or an immunosuppressive output (Meyer-Wentrup et al., 2008). Another study showed that antigen delivery to bone marrow stromal cell antigen 2 (BST2; CD317; tetherin) combined with TLR9-mediated activation induce strong humoral and cellular responses in mice against subsequent viral infections and tumor growth (Loschko et al., 2011). The cross-linking of the Ig-like RR ILT-7, which is a receptor to BST2, promotes the differentiation of pDCs into antigen presenting cells. The result induce T cell proliferation and activation in response to staphylococcal enterotoxin B indicating an immunogenic response (Tavano & Boasso, 2014). In conclusion, targeting antigen to pDC surface molecules can lead to either immunogenic or immunotolerant adaptive responses.

2.1.5. Regulatory receptors (RRs) and mechanisms of their function

PDCs express a series of Regulatory Receptors (RR) that serve to prevent aberrant immune responses and to regulate its major IFN-I-producing function apart from facilitating antigen capture (for review (Hirsch et al., 2017)). These include immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors such as the C-type lectin blood dendritic cell antigen 2 (BDCA-2; CD303; CLEC4C) (Dzionek et al., 2001), ILT-7 (CD85g) (Cao & Bover, 2010; Cao et al., 2006), or FcεRI (Schroeder et al., 2005), as well as natural cytotoxicity triggering receptor 2 (NCR-2; NKp44; CD336) (Fuchs, Cella, Kondo, & Colonna, 2005). Importantly, immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors need the association with adaptor molecules, such as DNAX activation protein of 12kDa (DAP-12), or the γ chain of Fc receptor ε (FcεRIγ), to initiate signal transduction. We also find immunoreceptor tyrosine-based inhibitory

motif (ITIM)-associated receptors such as the C-type lectin receptor DCIR (dendritic cell immunoreceptor) (Meyer-Wentrup et al., 2008) or CD300 (Ju et al., 2008). Other regulatory receptors are BDCA-4 (CD304; neurophilin-1), ILT-3 (CD85k), BTLA or DR6. After engagement, they signal through a B cell receptor (BCR)-like pathway that involves spleen tyrosine kinase (SYK), B cell linker protein (BLNK) and MEK-ERK/2 among others (Bao & Liu, 2013; Cao et al., 2007; Gilliet et al., 2008; Röck et al., 2007; Swiecki & Colonna, 2015) **(Figure 4)**. Signalling through this pathway diminishes the production of IFN-I and proinflammatory cytokines by an unknown mechanism (Aouar et al., 2016; Bao & Liu, 2013; Cao et al., 2006, 2007; Dzionek et al., 2001; Gilliet et al., 2008; Hirsch et al., 2010, 2017; Röck et al., 2007; Swiecki & Colonna, 2015). This regulatory mechanism can be used by viruses and some cancers to induce immune tolerance (Florentin et al., 2012; Hirsch et al., 2010; Martinelli et al., 2007; Woltman, Op den Brouw, Biesta, Shi, & Janssen, 2011; Y. Xu et al., 2009).

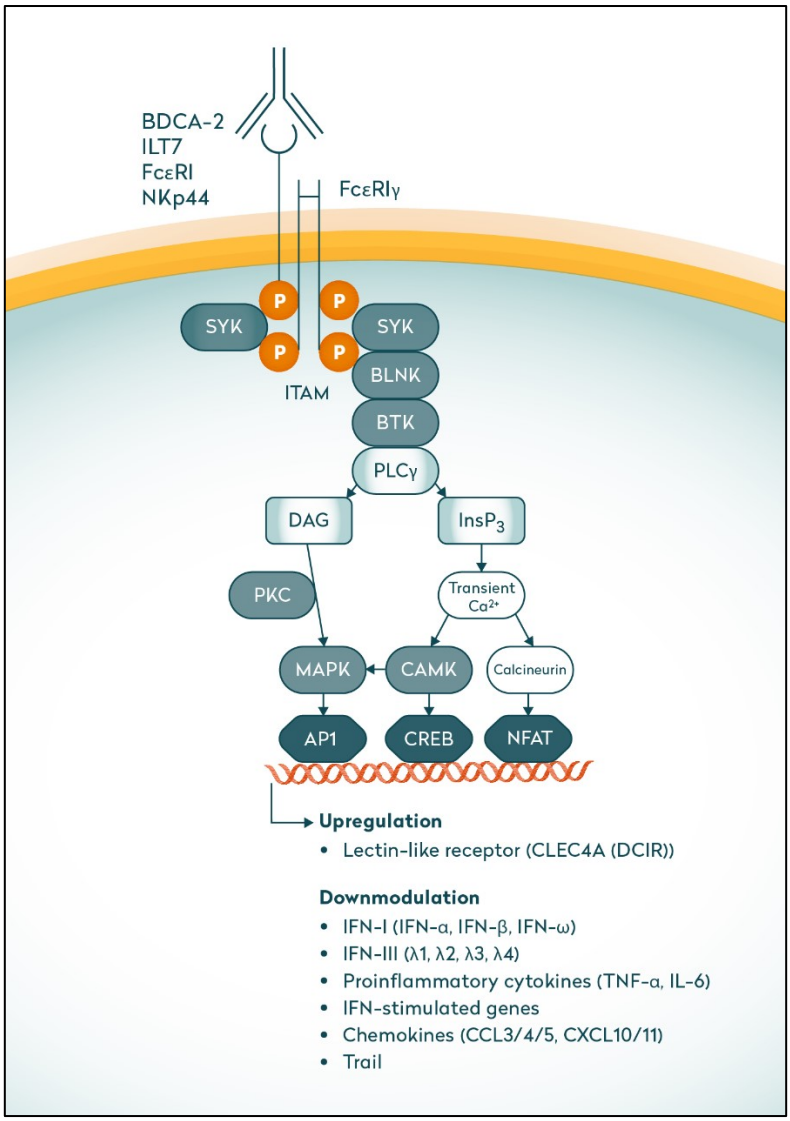

Figure 4: Regulatory signalling pathway. Crosslinking of regulatory receptors such as BDCA-2, ILT-7, FcεRI or Nkp44 triggers the activation of a signalling cascade that leads firstly to the phosphorylation of tyrosine residues within FcεRIγ ITAM region and recruitment of SYK. From this point, a BCR-like pathway involving BLNK, BTK and PLCγ results in the hydrolysis of PIP_2 into DAG and $InsP_3$. DAG activates PKC and the MAPKs, which ultimately activate the transcription factors AP-1 and NF-κB. In parallel, $InsP_3$ promotes transient internal release of Ca^{2+} followed by the activation of CAMK and the transcription factor CREB. Calcineurin leads to the activation of NFAT. *Adapted from Hirsch et al., 2017.*

BDCA-2, blood dendritic cell antigen 2; ILT-7, immunoglobulin-like transcript 7; FcεRI Fc epsilon RI/ high affinity IgE receptor; Nkp44: NCR-2, natural cytotoxicity triggering receptor 2; FcεRIγ, γ chain of Fc receptor ε; ITAM, immunoreceptor tyrosine-based activation motif; SYK, spleen tyrosine kinase; BLNK, B cell linker protein; BTK bruton´s tyrosine kinase; PLCγ, phospholipase C gamma; DAG, diacylglycerol; $InsP_3$, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; CAMK, calmodulin-dependent protein kinase; AP-1, activator protein 1; NF-κB, nuclear factor- κB; CREB, cyclic-AMP-responsive-element-binding protein; NFAT, nuclear factor of activated T cells.

2.1.6. Negative regulation of IFN-I production

Because of the destructive capacity of IFN-I on tissues, pDCs have evolved several mechanisms to negatively regulate its production. As mentioned above, RRs signalling is able to inhibit the pDC immunogenic response when bound to agonist antibodies (Aouar et al., 2016; Bao & Liu, 2013; Cao et al., 2006, 2007; Dzionek et al., 2001; Gilliet et al., 2008; Hirsch et al., 2010, 2017; Swiecki & Colonna, 2015). Importantly, being BDCA-2 exclusively expressed in pDCs makes of it an attractive target for immunomodulatory drugs **(Figure 5)**.

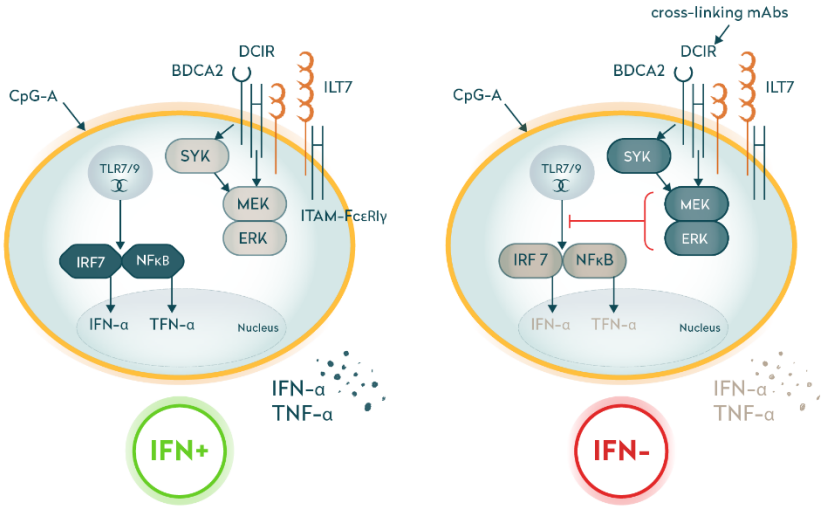

Figure 5: Effect of crosslinking of RRs on the production of type-I-IFN and proinflammatory cytokines. After TLR7/9 binding with its agonist a signalling pathway is triggered culminating in the translocation of IRF7, NF-κB and other transcriptions factors which induce the production of IFN-I/III as well as proinflammatory cytokines. If regulatory receptors are cross-linked with specific mAbs previously to TLR7/9 activation, TLR7/9 signalling and subsequently the production of IFN-I/III are inhibited.

RRs, regulatory receptors; CpG-A, class A CpG oligodeoxynucleotides; BDCA-2, blood dendritic cell antigen 2; DCIR, dendritic cell immunoreceptor; ILT-7, immunoglobulin-like transcript 7; TLR7/9, toll-like receptor 7/9; IRF7, interferon regulatory factor 7; NF-κB, nuclear factor κB; IFN-α, interferon α; TNF-α, tumor necrosis factor α; ITAM, immunoreceptor tyrosine-based activation motif; FcεRIγ, γ chain of Fc receptor ε; SYK, spleen tyrosin kinase.

A recently described mechanism of downregulation of IFN-I production involves the abundant surface receptors CXCR4 and CD44, which inhibit pDC activation when engaged with natural monoamines (N. Smith et al., 2017) and galectin-9 (Panda et al., 2018), respectively. Another pDC-mediated immunosuppressive mechanism involves IDO. This enzyme catabolizes

L-tryptophan to N-formyl kynurenine and has immunosuppressive effects because it depletes the tryptophan pool required by T cells to carry on effective responses. Also, receptor-type protein tyrosine phosphatase PTPRS, which is highly specific of pDCs within the immune system also participates as a negative regulator of pDC function. Studies showed that when PTPRS is deleted there is enhanced pDC activation, and when it is engaged with its ligand, pDC activation is inhibited (Bunin et al., 2015).

A recent study showed that the pleiotropic transcription factor Myc play an immunomodulatory role in pDC function. Myc was found to act as a repressor by binding the IRF7 promotor region and block its transcription. In experiments with GEN2.2 pDC cell line and Myc knock outs, production of Interferon stimulated genes was increased and further enhanced when CpG-B was added, triggering TLR9 signalling. Pharmacological targeting of Myc also recovered IRF7 expression, confirming the negative role of Myc in the regulation of pDC responses (T. W. Kim et al., 2016).

MicroRNAs are also involved in the regulation of pDC functions. Engagement of TLR7/9 induces the expression of miR-146a, which suppresses TLR-mediated signalling and NF-κB activation in pDCs (Karrich et al., 2013). MiR-155* (the complementary ssRNA to miR-155) is induced after TLR7 engagement and promotes type-I-IFN secretion by the inhibition of IL-1 receptor-associated kinase M (IRAKM). Its partner miR-155 is induced later and abolishes IFN-I production via the inhibition of TGF-beta-activated kinase 1 and MAP3K7-binding protein 2 (TAB2), a MAPKKK-interacting protein also called MAP3K7IP2 (Zhou et al., 2010). MiR-126 has also a role in the regulation of pDC . MiR126 targets mammalian target of rapamycin (mTOR) signalling pathway and regulates the expression of several important genes for pDC function, including *kdr* which encodes the growth factor VEGFR2. PDCs from $Kdr^{-/-}$ mice are not able to respond to TLR ligands, suggesting a role of VEGFR2 in IFN-I production regulation (Agudo et al., 2014).

Not less important is the capacity of hormones to regulate IFN-I production and pDCs function. It was shown that estrogen enhances TLR7-mediated signalling in pDCs (Seillet et al., 2012). That could provide an explanation for the higher capacity of IFN-I production by women pDCs in response to HIV compared to men pDCs (Meier et al., 2009).

2.1.7. Tolerogenic properties of pDCs

There is evidence that when unstimulated, pDCs promote immunosuppression when interacting with T lymphocytes by favoring the generation of T_{reg} cells, potent suppressors of T cell and DC activity (Matta, Castellaneta, & Thomson, 2010). The poor stimulatory capacity of naïve pDCs may be related to the rapid turnover of MHC class II due to the persistently expressed MHC class II ubiquitin E3 ligase RING-CH1 (MARCH1) (Ishido, Matsuki, Goto, Kajikawa, & Ohmura-Hoshino, 2010), which ubiquitinates MHC class II molecules labelling them for internalization and degradation. Since the lack of antigen on the cell surface promotes the generation of T_{regs}, it is possible that the fast internalization of antigen-MHC class II complexes implies low antigen presentation and so the promotion of T_{regs} maturation and function when interacting with T cells (Molinero, Miller, Evaristo, & Alegre, 2011; Turner, Kane, & Morel, 2009; Young et al., 2008).

In pDCs, IDO facilitates the maturation of naïve $CD4^+$ T cells into T_{regs} through CD40/CD40L-mediated signalling (Fallarino et al., 2005). Thus, IDO expressed by pDCs has a tolerogenic function under physiological conditions, essential to prevent aberrant immune reactions such as autoimmune reactions or to facilitate maternal-fetal recognition during pregnancy (Fallarino, Gizzi, Mosci, Grohmann, & Puccetti, 2007; Mellor & Munn, 2004). In addition to the aforementioned mechanisms, the expression of ICOS-L on pDCs can promote the maturation of T cells into IL-10 producing T_{regs}, highlighting the tolerogenic ability of these cells (Ito et al., 2007). Importantly, direct engagement of IL-10 as well as transforming growth factor beta (TGF-β) by pDCs have been shown to decrease IFN-I secretion in response to TLR ligands (Duramad et al., 2003; L. Li et al., 2008). Another mechanism by which pDCs can mediate immunotolerance is through the upregulated expression of the immunosuppressive molecule PD-L1, which interacts with the receptor PD-1. After interaction, these molecules induce the blockade of the t cell receptor (TCR)-induced stop signal in the target T cell (Fife et al., 2009), thus, promoting peripheral tolerance. PDCs can also supress the proliferation of effector T cells by the expression and release of granzyme B in response to IL-3 (Jahrsdorfer et al., 2010).

2.1.8. Virus recognition by pDCs

During the last two decades, several studies shed light into the way viral components reach the TLRs and trigger IFN-I response. So far, investigations showed that there is not only one way in which viral recognition takes place but multiple options. Direct recognition through TLRs of internalized virus that do not infect pDCs was supported by the capacity of pDCs of responding to inactivated virus or to replication-deficient virus **(Figure 6A)** (Asselin-Paturel et al., 2001; Deal, Jaimes, Crawford, Estes, & Greenberg, 2010; Kumagai et al., 2009; Lund et al., 2003). The fact that pDCs are highly resistant to productive viral infection by some virus, such as Newcastle disease virus (NDV) (Kumagai et al., 2009), to which they can respond to with IFN-I production also supports this thesis. Other viruses, such as vesicular stomatitis virus (VSV) can induce type-I-IFN production only if they are replication-competent **(Figure 6B)** (Hornung et al., 2004; Lee, Lund, Ramanathan, Mizushima, & Iwasaki, 2007). Productive infections of pDCs by several virus, such as Coronavirus, Myxoma virus, Paramyxovirus or Arenavirus have been reported *in vitro* (Cervantes-Barragan et al., 2007; Dai et al., 2011; Manuse, Briggs, & Parks, 2010) and *in vivo* (Macal et al., 2012). Notably, VSV elicits a more robust IFN-I response when pDCs recognize VSV-infected cells rather than by direct interaction with free virions (Frenz et al., 2014). This is also the case of other viruses, such as HCV, in which only the recognition of HCV-infected hepatocytes elicit a significant IFN-I response (Dreux et al., 2012; Florentin et al., 2012; Takahashi et al., 2010) This mechanism of virus recognition is TLR-7-dependent and lymphocyte function-associated antigen 1 (LFA-1)-mediated adhesion or exosomal transfer of viral components is necessary **(Figure 6C)** (Assil et al., 2019; García-Nicolás et al., 2016; Saitoh et al., 2017; Tomasello et al., 2018). Subsequent studies demonstrated that several RNA viruses are mainly recognized in the same manner, including retroviruses (Lepelley et al., 2011; Rua, Lepelley, Gessain, & Schwartz, 2012), lymphocytic choriomeningitis virus (LCMV) (Wieland et al., 2014), hepatitis A virus (HAV) (Z. Feng et al., 2015), dengue virus (DENV), west Nile virus (WNV) (Decembre et al., 2014) and yellow fever virus (YFV) (Bruni et al., 2015). In Epstein-Barr virus (EBV)-latently infected cells, this mechanism can be observed when EBV-derived RNA is transferred to pDCs through exosomes and triggers the induction IFN-I and subsequently ISGs **(Figure 6C)** (Baglio et al., 2016). It has been suggested this mechanism of recognition of an infected cell may occur also in non-viral pathogens like malaria. In this regard, close interactions between infected macrophages and pDCs have been observed during TLR7-mediated activation of pDCs in malaria-infected mice (Spaulding et al., 2016).

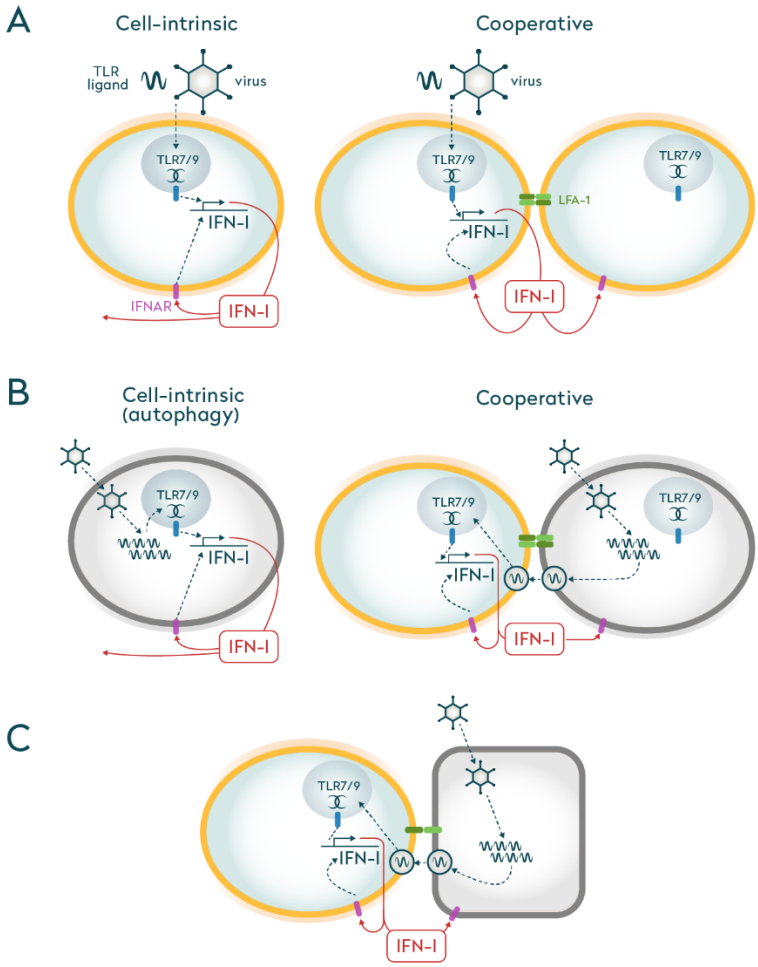

Figure 6: Scheme of the different models of virus recognition by pDCs. Infected cells are characterized in grey. (A) Direct recognition of viruses that do not infect pDCs. Depicted in the traditional cell-intrinsic model and in the cooperative model, which requires cell to cell contact. In the cooperative model, LFA-1-mediated adhesion is necessary for pDCs to elicit an interferon response. (B) Recognition of viruses that can infect and replicate within pDCs. The cell-intrinsic model is based in autophagy-mediated TLR signalling (see below) and in cooperative model, the cell that starts the IFN-I response is not the one that is infected, but the adjacent one. Virus-derived TLR ligands can reach other cells via exosomes. (C) pDCs sense virus from infected cells of different types (e.g. hepatocytes) via exosomes or by cell-cell contact through viral synapses. *Adapted from Reizis, 2019*.

TlR7/9, toll-like receptor 7/9; IFN-I, type I interferon; IFNAR, interferon-α receptor; LFA-1, lymphocyte function-associated antigen 1.

Viruses and nucleic acids can also be internalized in the cell via Fc receptors, which work as RRs, if they are bound to antibodies during an immune response. Sendai virus immune

complexes interact with the inhibitory Fc receptor FcγRIIB, which promotes the uptake of the complexes, thus, preventing an IFN-I response against the virus, (Flores et al., 2015; Hirsch et al., 2017). On the contrary, a complex of antinuclear antibodies with endogenous nucleic acids can interact with the activating Fc receptor FcγRIIA and promote IFN-I production after internalization (Barrat et al., 2005; Bave et al., 2003; Means et al., 2005).

Autophagy also plays a role in viral recognition and may be a mechanism by which a virus replicating inside can gain access to endosomal compartments **(Figure 6B)**. For example, ssRNA recognition by TLR7 requires transport of cytosolic viral intermediates into the lysosome by autophagy (Lee et al., 2007).

2.1.9. TIM-3 as a marker of pDC dysfunction

T cell Ig and mucin-domain containing molecule-3 (TIM-3) is expressed in several cell types, including cells of the lymphoid and myeloid lineages, and it is involved in the regulation of several cellular functions (Han, Chen, Shen, & Li, 2013). TIM-3 has been shown to be an activation and exhaustion marker of $CD4^+$ and $CD8^+$ T cells (Anderson, Joller, & Kuchroo, 2016; R Brad Jones et al., 2008). Importantly, TIM-3 function seems to be cell type specific and in 2015 it was identified by Gurka et al., as a surface molecule on pDCs (Gurka, Dirks, Photiadis, & Kroczek, 2015). In a recent study, J. A. Schwartz et al., howed that in vitro-activated pDCs expressing TIM-3 were defective in type-I-IFN and TNF-α production and found a direct correlation between the strength of the pDC stimulus and TIM-3 expression and the subsequent dysfunction. The dysfunction is associated with the recruitment of IRF7 and p85 into lysosomes and with the submembrane displacement of TLR9 (J. A. Schwartz et al., 2017). Therefore, the study sets TIM-3 as a marker of pDC dysfunction.

2.1.10. PDC role in cancer

IFNs-I/III have not only antiviral properties but also antitumoral activity and are used as a treatment in different types of cancer including both hematological and solid tumors. It has been shown that IFN-I affects tumor cell proliferation, metastasis, and tumor lymph/angiogenesis (reviewed in Demoulin, Herfs, Delvenne, & Hubert, 2013). Being the major type-I-interferon producers, pDCs are thought to have an important role in the cancer context and it is expected that a dysregulation of pDC function can lead to immunodeficient states or inefficient immune

responses to tumors. Against the expectations, recruitment of pDCs to tumor localization is associated with bad prognostic and immunosuppression (Treilleux et al., 2004). PDCs recruited to tumors receive the name of tumor-associated pDCs (TApDCs) and upon challenge with TLR9 agonists, produced low amounts of IFN-α. Notably, a positive correlation between tumor aggressivity and the frequency of TApDCs has been reported. Subsequent studies also have shown a correlation between the frequency of TApDCs and the frequency of T_{regs} (X.-M. Huang et al., 2014; Pedroza-Gonzalez et al., 2015; Sisirak et al., 2012). Thus, pDCs, which infiltrated breast and ovarian tumors have been shown to produce low quantities of IFN-I but have an increased capacity to induce the development of T_{regs} (Conrad et al., 2012; Hartmann et al., 2003; Labidi-Galy et al., 2011; Pedroza-Gonzalez et al., 2015; Sisirak et al., 2012). TApDCs are able to induce the production of IL-10 *via* T_{regs} and naïve $CD4^+$ T cells *via* expression of ICOS-L and contribute in this way to immunosuppression (Faget et al., 2012; Ito et al., 2007; Pedroza-Gonzalez et al., 2015). Aberrant function in pDCs may be caused by tumor-associated soluble factors, such as TGF-β and TNF-α and probably also by IL-10 produced by T_{regs}. Recent studies with genetically depleted pDCs in mice supported this idea (Terra et al., 2018). Alternatively, BST-2 expressed by tumor stroma inhibits pDCs *via* ILT-7 engagement. (Cao et al., 2009; Hirsch et al., 2010; Swiecki & Colonna, 2015). An indirect effect of the low type-I-IFN production by TApDCs is the loss of activity of natural killer (NK) cells, which normally are important antitumor agents (Rautela et al., 2015). In an orthotopic mouse mammary tumor model, the functionality of TApDCs was found to be altered in response to TLR9 agonists and *in vivo* depletion of TApDCs delayed tumor growth. Contrarily, when TApDCs were activated via *in vivo* administration of TLR7 ligand, tumor regression through an IFN-I mediated mechanism was observed (Le Mercier et al., 2013). Interestingly, breast and ovary cancer patients showed a decrease in the number of circulating pDCs which correlated with an increase of tumor-infiltrating pDCs, indicating the migration of pDCs from blood to the tumor location (Labidi-Galy et al., 2011; Sisirak et al., 2012).

2.1.11. PDC role in autoimmune diseases

Since it was known that pDCs were able to initiate such a strong inflammatory state, about twenty years ago, the suggestion of a potential role in autoimmune diseases soon was made (Ronnblom & Alm, 2001). Indeed, by 2020 several studies demonstrated the importance of pDC function in almost all autoinflammatory diseases. The most well-known case of the involvement of pDCs in the pathogenesis and mechanism of an autoimmune disease is in SLE. In SLE, a self-directed immune response is caused by the generation of immune complexes of autoantibodies

and nucleic acid-containing nuclear antigens (for review Panda, Kolbeck, & Sanjuan, 2017). An interferon signature is found in more than half of SLE patients, and the deletion of IFNAR has been shown to reduce SLE in experimental models. Also, the clinical manifestation of SLE correlates with the redistribution of pDCs from blood to lymphoid tissue. Importantly, IFN-I professional producer pDC subset (P1-pDCs, according to Alculumbre et al.), was detected in samples of psoriasis and SLE patients (Alculumbre et al., 2018). Molecular features associated with SLE induced IFN-I production by pDCs, and the depletion of pDCs reduced significantly SLE symptoms in different experimental models (Caielli et al., 2016; Davison & Jorgensen, 2015; Lood et al., 2016; Rowland et al., 2014; Sisirak et al., 2014). In diabetes, it has been reported an expansion (Allen et al., 2009) and infiltration of pDC into pancreatic islets in a model of autoimmune-prone non-obese diabetic (NOD) mice (Diana et al., 2013). Also, a selective depletion of pDC correlated with an improvement of the clinical status of the NOD mice (Hansen et al., 2015). In scleroderma (SSc), which targets connective tissue, patients showed an IFN signature and pDCs produced type-I-IFN in response to pathogenic immune complexes (Eloranta et al., 2010; D. Kim et al., 2008). High levels of the proinflammatory chemokine CXCL4 produced by pDCs are also involved in SSc pathogenesis and correlate with disease progression (van Bon et al., 2014). A recent study reported that pDCs from SSc patients express TLR8, contrarily to pDCs from healthy donors. This study linked TLR8 signalling in pDCs with the induction of CXCL4, showing also that CXCL4 exacerbates TLR-mediated IFN-α production (Ah Kioon et al., 2018). More ambiguous roles of pDC have been reported in other autoimmune diseases like psoriasis, arthritis or atherosclerosis. Regarding psoriasis, experiments with genetic models have shown a mild stage-specific role of pDCs (Glitzner et al., 2014). In an antibody-mediated model or arthritis, depletion of pDCs favored the progress of the disease (Nehmar et al., 2017). In a model of atherosclerosis, conflicting results from two different teams concluded that pDC ablation promote (Yun et al., 2016) or impair (Sage et al., 2014) the disease. On the other hand, in inflammatory bowel disease, which is caused by an aberrant immune response to microbial components of the gastrointestinal tract, pDC ablation had no effect in the output compared to controls (Sawai et al., 2018).

The localization of TLRs in endosomes probably aims to prevent unwanted activation by self-nucleic acids. It is important to note that the increased expression of either TLR7 or huTLR8 in transgenic mice is sufficient to induce autoimmunity. Thus, TLR7/9 in pDCs, as well as TLR8 expression is not only restricted in endosomes as a mean of protection but it is also tightly regulated (Deane et al., 2007; Guiducci et al., 2013; Walsh et al., 2012).

Besides autoimmune diseases, pDCs have been shown to play an important role in allergic processes such as allergic dermatitis, allergic rhinitis and asthma. pDCs have been shown to be recruited in the nasal mucosa of allergic patients, and to be involved in allergic inflammation (Dua, Watson, Gauvreau, & O'Byrne, 2010; Jahnsen et al., 2000). Evidence points to a favoring of a Th2 response by pDC as a mechanism for allergy. A study showed that in healthy individuals, IFN-I secreted by pDCs after exposure to rhinovirus limit the Th2-driving response (Pritchard et al., 2012). It has been suggested that in allergic patients this mechanism may be defective, as an explanation for exacerbated asthma triggered by viral infections (Froidure, Vandenplas, D'Alpaos, Evrard, & Pilette, 2015).

2.1.12. PDCs in bacterial, fungal and parasitic infections

The role of pDCs in the context of infections by other pathogens than virus has been much less studied, and so the mechanism of activation of pDCs by these pathogens as well as their fate are still unclear. It is known that pDCs can respond to gram-positive and gram-negative bacteria by upregulating co-stimulatory molecules and producing IFN-I (Michea et al., 2013). They have been demonstrated to play a role also in fungal infections. In the presence of *Aspergillus fumigatus hyphae*, pDCs function as immunogenic agents by secreting IFN-I and TNF-α (Ramirez-Ortiz et al., 2008). Very interestingly, they contribute to the killing of the fungi by directly secreting molecules that chelate divalent cations, such as calprotectin and lactoferrin, which are necessary for fungus to grow (Ramirez-Ortiz et al., 2011). In the presence of *A. fumigatus*, pDCs are also capable to secrete pDC extracellular traps (pETs) in a similar manner that neutrophils secrete neutrophil extracellular traps (NETs) (Goldmann & Medina, 2012). These traps, which are formed by DNA and citrullinated histone H3, assemble with antimicrobial peptides and serve to kill microbes that cannot be phagocytised (Loures et al., 2015). Regarding parasitic infections, malaria parasite *(Plasmodium falciparum)* does not activate pDCs in its blood-stage (Loughland et al., 2017). However, experiments with mice showed that TLR7-mediated pDC activation can have a protective role in the initial phases of infection (X. Yu et al., 2016).

2.1.13. GEN2.2 pDC cell line

Due to the low numbers of pDCs in peripheral blood, the studies involving primary pDCs are not exempt of difficulties. To date, few pDC cell lines have been developed. Among them we find the leukemia-derived PMDC05 (Narita et al., Blood 2005), the cell line CAL-1 from a blastic NK cell lymphoma origin (Maeda et al., Int J Hematol 2005) and GEN2.2 (Chaperot et al., J Immunol 2006), generated from a BPDCN (L Chaperot et al., 2001). In our study we used GEN2.2 as a pDC model. GEN2.2, like pDCs, present on their surface the phenotypic markers CD4, HLA-DR, HLA ABC, CD45RA and CD123. They are negative for the myeloid markers CD13, CD11b, CD11c, CD14 and CD64. Importantly, they express also BDCA-4 and the specific pDC marker BDCA-2. They also express high levels of CD86 and moderate levels of CD40, whereas there is no sign of CD80 on its surface. Regarding homing and chemokine receptors, they are positive for CXCR3, CXCR4 and CD62L and express weakly CCR5, CCR6, CCR7 and CXCR2 whereas they show no expression of CCR1, CCR2, CCR4 and CXCR1 (Chaperot et al., 2006). Very importantly, they are expressing the endosomal receptors TLR7 and TLR9. GEN2.2 cells are growing in suspension and they are slightly adherent. They need a feeder layer of MS-5 cells (a murine stromal cell line) to grow. GEN2.2 cells produce massive quantities of IFN-α after engagement of TLR7 with influenza virus, but not the TLR7 synthetic agonist R878 (Resiquimod), and TLR9 with HSV-1 or 2 or the TLR9 synthetic agonist CpG-A. On the other hand, when they are activated with CpG-B, IL-3+CD40L or other viruses, GEN2.2 maturate to an APC phenotype, secreting few amounts of IFN-α but considerable levels IL-1, IL-6 and TNF-α to the media. (Chaperot et al., J Immunol 2006).

2.2. Human immunodeficiency virus type I

Human immunodeficiency virus type I (HIV-1) is a lentivirus under the category of retroviruses that is well-known to establish a chronic infection in humans. If untreated, HIV-1 leads to a severe immunodeficiency, the acquired immunodeficiency syndrome (AIDS), that may ultimately lead to the dead of the infected patient due to opportunistic infections or cancer (Barré-Sinoussi, Ross, & Delfraissy, 2013).

2.2.1. HIV transmission

HIV-1 has been isolated from many human liquids and secretions, including blood, saliva, urine, pre-ejaculate, semen, tears, vaginal fluids, breast milk, amniotic fluid, and cerebrospinal fluid, among others. Even though the virus is present in almost any human tissue or secretion, there are only three reported mechanisms of transmission, namely, sexual transmission, blood-blood contact or vertical transmission (mother to child) pre-birth, during birth or breast-feeding. Sexual transmission occurs when sexual contact exists without protection between a seronegative individual and a seropositive individual who does not receive ART. Transmission is produced by contact of infected blood or secretions of genital mucosa, rectal mucosa or oral mucosa. Transmission via blood occurs through contaminated needles (drug users sharing infected needles, corporal modifications like piercings, tattoos or scarification or laboratory accidents with infected material) or during a defective blood transfusion **(Table 1)**. It is important to note that the aforementioned modes of transmission happen only when the infected individual is not under antiretroviral treatment. Of these, transmission during birth used to be the most problematic. Nowadays, it is totally under control in developed countries due to the administration of highly active antiretroviral treatment (HAART), specially indicated for these cases, since before or at the beginning of pregnancy. Delivery is performed by Caesarean, maternal breast-feeding is substituted, and the baby starts antiretroviral treatment from the moment of birth.

Estimated risk of VIH acquisition depending on the type of exposition (D. K. Smith et al., 2005)	
Type of exposition	**Estimated number of infections per each 10000 expositions to an infected source**
Blood transfusion	9000 (Donegan et al., 1990)
Birth	2500 (Coovadia, 2004)
Drug injection	67 (Kaplan & Heimer, 1995)
Receptive anal intercourse*	50 (“Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on Heterosexual Transmission of HIV.,” 1992) (Varghese, Maher, Peterman, Branson, & Steketee, 2002)
Laboratory needle	30 (Bell, 1997)
Receptive vaginal intercourse*	10 (“Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on Heterosexual Transmission of HIV.,” 1992; Leynaert, Downs, & de Vincenzi, 1998; Varghese et al., 2002)

Insertive anal intercourse*	6,5 (“Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on Heterosexual Transmission of HIV.,” 1992; Varghese et al., 2002)
Insertive vaginal intercourse*	5 (“Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on Heterosexual Transmission of HIV.,” 1992; Varghese et al., 2002)
Receptive fellation*	1 (Varghese et al., 2002)
Insertive fellation*	0,5 (Varghese et al., 2002)

Table 1: Data from several studies showing the number of infections per each 10000 expositions to an infected source. ** without the use of condom.*

Importantly, a series of recent studies have confirmed that HIV is not transmitted when the infected individual is under ART and the viral load is under the limit of detection of commercial assays (< 50 copies of viral RNA/ml). This is known as “U=U” or “Undetectable=Untransmissible” or “can´t pass it on” (Eisinger, Dieffenbach, & Fauci, 2019; Hiv, 2017). The studies demonstrating this hypothesis are: Opposites attract (Bavinton et al., 2018), PARTNER 1 (Rodger et al., 2016), PARTNER 2 (Rodger et al., 2019), and HPTN052 (M. S. Cohen et al., 2016). Taking the results of these studies together, from 4097 couples from 4 continents and 151880 sexual acts without condom, there were zero HIV transmissions where the viral load was under the level of detection. The affirmation that “undetectable viral load makes HIV untransmissible” gives new hopes to the eradication of HIV pandemic by preventing HIV transmission.

2.2.2. HIV-1 particle structure

HIV-1 virion is round, measuring around 100 nm in diameter and it is composed of a lipidic envelope, a structural matrix and a conical-shaped capsid that contains a pair of identical positive-sense single-stranded RNA molecules as well as essential enzymes for replication (Reverse Transcriptase (RT)/RNase H and integrase (IN)) **(Figure 7)**. These essential enzymes are bound to the nucleic acid chains, which are associated with the nucleocapsid protein (NU). The conical capsid is formed by the assembly of the inner capsid protein p24 (CA), and a symmetrical outer capsid is formed by the matrix protein p17 (MA) (Niedrig et al., 1994). The host-derived envelope is a lipid bilayer containing 72 trimers of the viral envelope surface protein, each one of them composed by the surface protein gp120 (SU) and the transmembrane protein gp41 (TM)

(Gelderblom, 1991), as well as host membrane proteins derived from the budding. Several units of viral protease (PT) are found in the region between the matrix and the capsid. The maturation of the virion occurs at the end of the budding process when the precursor proteins Gag and Gag/Pol (p55, p160) are cleaved into individual units (For review: Sundquist & Kräusslich, 2012).

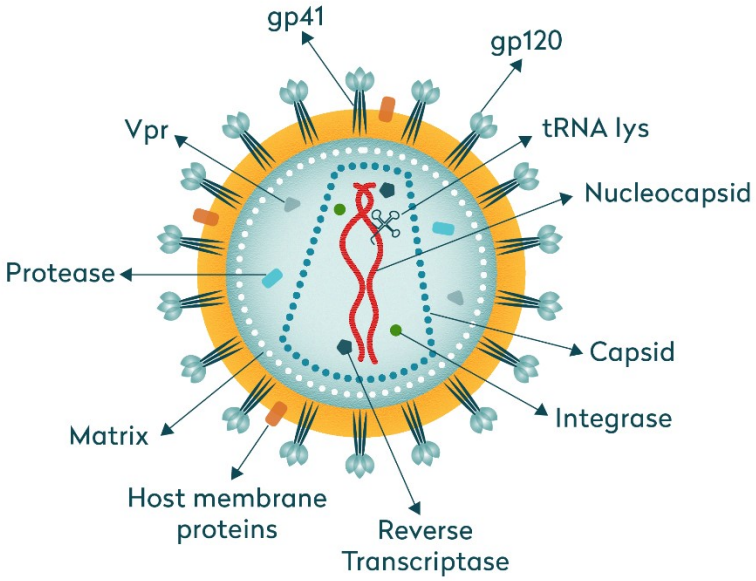

Figure 7: Scheme of HIV-1 virion structure. HIV-1 virion envelope is composed of a lipidic bilayer acquired during the budding process from the host cell along with host membrane proteins and the surface viral protein Env. Env protein is composed of gp120 and gp41 arranged in trimers. The matrix, adjacent to the internal lipidic layer, provides a scaffold to the whole structure. The characteristic cone-shaped capsid of HIV-1 contains 2 copies of a 9.8 kb (+) ssRNA within the nucleocapsid as well as the essential viral enzymes RT and IN and also accessory viral proteins. In the space left between the matrix and the capsid there are viral proteases.

Env, envelope; RT, reverse transcriptase; IN integrase; Vpr, viral protein r.

2.2.3. Genome organization

HIV-1 genome is formed by two identical molecules of positive sense ssRNA of 9.8 kb in length. Once integrated into the host genome, the pro-viral dsDNA become flanked by long terminal repeats (LTRs). HIV-1 genome comprises 9 genes, 3 of which are the common retroviral *gag-pol-env* and 6 other accessory genes that are unique to HIV-1 **(Figure 8)**. These genes are *vif, vpr, tat, rev, vpu,* and *nef.* The genome is organized in 3 open reading frames (ORFs). *Gag*

leads the synthesis of the precursor protein gag, which is processed by PT to form the inner structural proteins of the nucleocapsid, capsid, and matrix of the virion.

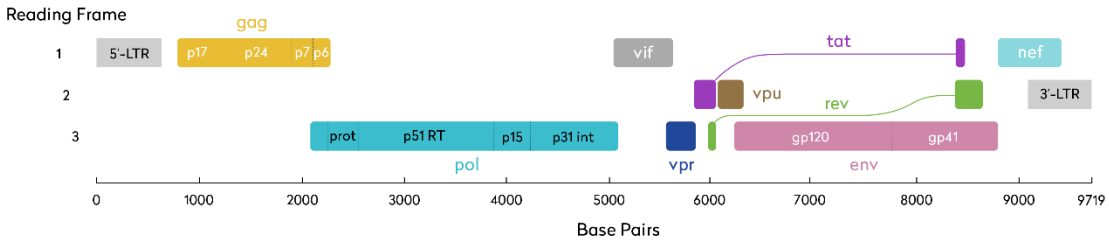

Figure 8: HIV-1 genome organization. HIV-1 genome is 9.8 kb long and is encoded in all three reading frames, indicated by the overlaps. The viral genome contains nine genes that codify for fifteen different proteins. Gag, pol and env are common to all retroviruses and the accessory genes vif, vpr, vpu, tat, rev and nef are unique to HIV. HIV-1 mRNA undergoes differential splicing to obtain nine different gene products. The 9.1 kb unspliced genomic transcript encodes for Gag and Pol precursor polyproteins. Five singly spliced transcripts with an average length of 4.3 kb encode for Env and the accessory proteins Vif, Vpr and Vpu. Three multiply spliced mRNAs encode for Nef and the regulatory proteins Tat and Rev. *Adapted from the original by Thomas Splettstoesser (https://commons.wikimedia.org/w/index.php?curid=33943759).*

LTR, long terminal repeat; Gag; structural protein-encoding transcript; Pol, enzymatic protein-encoding transcript; Env, envelope protein-encoding transcript; Vif, viral infectivity factor; Vpr, viral protein r; Vpu, viral protein u; Tat, trans-activator of transcription; Rev, regulator of expression of viral proteins; Nef, negative regulatory factor.

The gene *pol* encodes the viral enzymes that are essential to complete the viral cycle. These proteins are formed by posttranslational cleavage of Pol polyprotein into the RT, which reverse transcribes the genomic RNA into cDNA, IN, which integrates the viral reverse-transcribed DNA within the host DNA, and the PT, which, as mentioned above, is implied in the cleavage of the precursor polyprotein of the structural peptides that will form the mature virion. The *env* gene codes for the precursor glycoprotein gp160, which is cleaved by cellular proteases to give gp120 (SU) and gp 41 (TM). The accessory genes *vif, vpr, tat, rev, vpu,* and *nef* code for multifunctional proteins aimed to target a large variety of cellular antiviral factors involved in innate and adaptive immune response, including the restriction factors SAMHD1, APOBEC3B and BST-2. These accessory proteins not only act at a protein level but also suppress the expression of antiviral factors by modulating the activity of immune-regulatory transcription factors such as NF-κB. They exert also a regulatory function on viral genes (Buffalo, Iwamoto, Hurley, & Ren,

2019; Malim & Emerman, 2008; Ramirez et al., 2019; Sauter & Kirchhoff, 2018; Sauter et al., 2009).

2.2.4. HIV-1 replicative cycle

HIV-1 is either sexually transmitted or transmitted by blood contact between two individuals, but non-sexual transmission can happen by an infected mother to her infant during pregnancy, birth or after birth through the breast milk. After entering the human body the virion attaches to CD4 molecules present on the surface of $CD4^+$ leukocytes (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986). Recent investigations suggested that the primary target in men may be not $CD4^+$ T cells or dendritic cells but urethral macrophages (Ganor et al., 2019). The co-receptors CXCR4 and CCR5 are also essential for the attachment and fusion with the cell membrane (Y. Feng, Broder, Kennedy, & Berger, 1996; Weiss, 2013). After the fusion of the viral envelope and the plasma membrane of the target cell, the nucleocapsid with the genetic material as well as the proteins accompanying the virion (RT, IN, and PT) are released into the cytosol and transported *via* microtubules to the nucleus, in the form of the pre-integration complex (PIC) **(Figure 9)**. During the microtubule-based transport of PIC the viral RNA is reverse transcribed to cDNA and once within the nucleus it is integrated into the host genome with the aid of the viral integrase (Chan & Kim, 1998; Wilen, Tilton, & Doms, 2012).

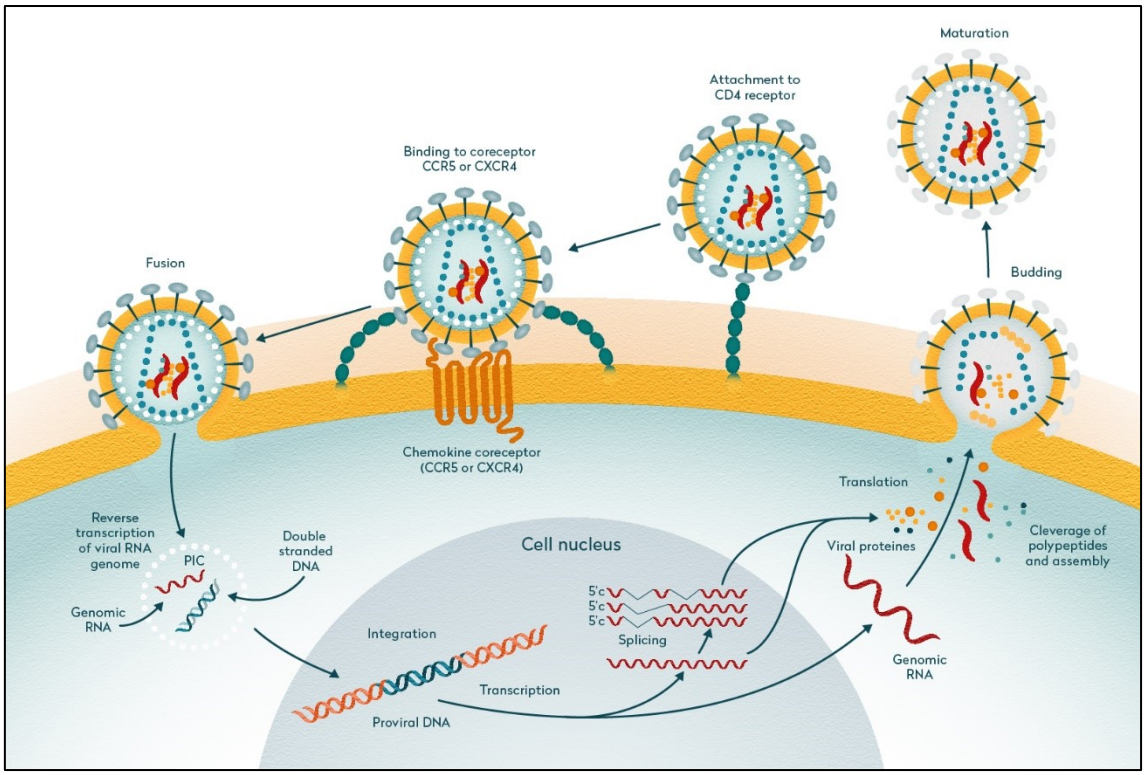

Figure 9: HIV replicative cycle. HIV gp120 attaches CD4 surface protein and with the aid of the chemokine receptor CCR5 or CXCR4 the membrane of the virion fuses with the membrane of the target cell. The PIC, which includes HIV RNA, integrase, reverse transcriptase and other viral proteins enters the cell. RNA is reverse transcribed to DNA by the viral reverse transcriptase and it is transported to the nucleus where the viral integrase catalyzes the integration into the host DNA. Viral RNA is generated by the host´s RNA pol II, spliced to produce early proteins Nef, Tat, Rev. Later, by means of the regulatory protein Rev the spliced and unspliced transcripts are exported from the nucleus and used either to be translated into viral proteins or as genomic RNA. Recently made viral polyproteins Gag-Pol and viral genomic RNA concentrates in hotspots by the cell membrane where glycosylated Env protein is present and an immature particle buds from the host cell. The new virion is released, and the viral protease cleaves the polyprotein in order to get the mature viral particle.

PIC, pre-integration complex; CCR5, CC-chemokine receptor 5; CXCR4, CXC-chemokine receptor 4; Gag; structural protein-encoding transcript; Pol, enzymatic protein-encoding transcript; Env, envelope protein-encoding transcript; Tat, trans-activator of transcription; Rev, regulator of expression of viral proteins; Nef, negative regulatory factor.

2.2.5. HIV Pathogenesis

Transmission of HIV-1 is usually established through epithelial surfaces covering genital mucosa by a transmitted/founder (T/F) virus with unique features. As an example in a model of

human foreskin epithelium, highly HIV-1-infected mononuclear cells in polarized exposure to the inner but not outer foreskin are able to carry on an efficient HIV-1 transmission event. These HIV-1-infected mononuclear cells form viral synapses with foreskin keratinocytes, promoting polarized budding of HIV-1 which is rapidly internalized by Langerhans cells. Finally, Langerhans cells form conjugates with T cells, thus, transferring HIV-1. For a detailed review (Bruxelle, Trattnig, Mureithi, Landais, & Pantophlet, 2021; Joseph, Swanstrom, Kashuba, & Cohen, 2015).

The defining characteristics of the transmitter/founder virus are driven by selective pressure. Studies showed that T/F viruses present enhanced binding to target cells due to a more accessible CD4-binding site within env. Regarding the possibility of a higher env density on the membrane of T/F viruses that would increase the chance of binding to a target cell studies showed discordant results. Since initial studies showed that T/F viruses might have a higher number of env a later report concluded the contrary. HIV-1 T/F viruses predominantly use CCR5 as correceptor along with CD4 (Bruxelle et al., 2021; Grivel, Shattock, & Margolis, 2011). However, CXCR4- tropic HIV-1 T/F viruses have been observed, as well as CXCR6-tropism in the case of mother-to-child transmission. Other characteristics of T/F HIV-1 viruses are enhanced replicative capacity and the ability to evade primary innate immune response, specially through resistance to IFN-I antiviral response. T/F viruses have been found enhanced interaction with DCs (Parrish et al., 2013).

The primary target of HIV are CD4+ T lymphocytes but other cells displaying CD4 and the chemokine receptors can also be infected, including dendritic cells, macrophages, monocytes and resting T lymphocytes. Importantly, a recent investigation involving penile tissue from HIV-1-infected individuals under antiretroviral therapy (ART) showed that urethral macrophages contain integrated HIV-1 DNA, RNA, and intact virions. Moreover, HIV-1-infected urethral macrophages are able to produce replication-competent HIV-1 virions after activation. Therefore, skin-resident macrophages are not only important for transmission to T-cells in the form of conjugates, but this study sets them as a new recipient for the HIV-1 reservoir (Ganor & Bomsel, 2011; Ganor et al., 2019).

CD4-independent infections have been also reported, such as infection of astrocytes (Y. Liu et al., 2004), enterocytes or infection of renal epithelial cells (Chen et al., 2011), which can later give rise to HIV-associated cognitive disorder and nephropathy, respectively. After primary infection takes place, a rapid burst in HIV replication and viral load happen, followed by a pronounced decrease of $CD4^+$ T cells **(Figure 10)**. $CD4^+$ T cells depletion is especially noticeable in the intestine, where HIV-1 pathogenesis begins. A potent induction of proinflammatory

cytokines and chemokines occurs in response to the presence of viral antigens (Stacey et al., 2009). The decrease of $CD4^+$ T cells is due not only to the cytolytic effects of HIV-1 replication but also to the action of HIV-specific $CD8^+$ T cells. A few weeks later, viral load diminishes to a set-point, which is maintained by both innate and adaptive immunity, and $CD4^+$ T cell numbers are partially restored. Because of the high mutation rate of HIV-1 genome, the strong adaptive response to the virus selects mutations in the key epitopes, thus leading to immune escape (Goonetilleke et al., 2009).

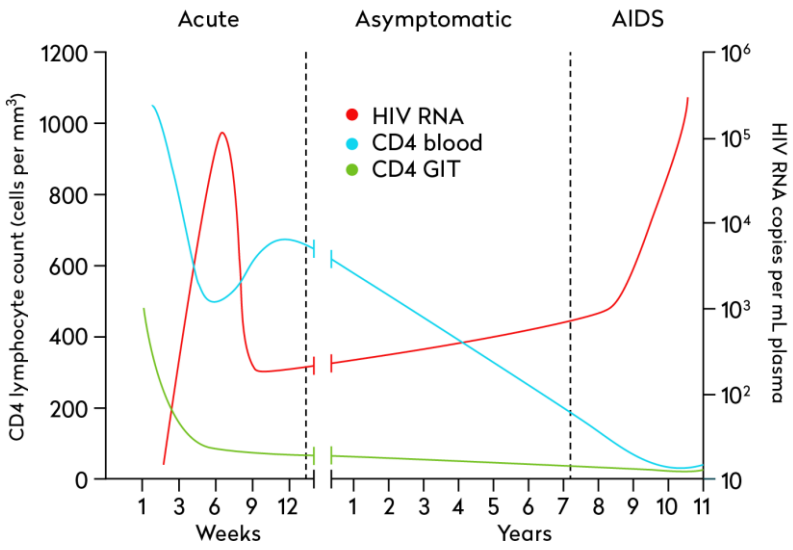

Figure 10: HIV-1 load and $CD4^+$ T cells dynamics during HIV-1 infection. In untreated HIV-infected patients $CD4^+$ T cells are depleted progressively from blood and reduced almost completely from gastrointestinal tract (GIT) few weeks after infection. HIV RNA in plasma increases dramatically during the first weeks of infection and later decreases up to a set point after HIV-specific adaptive immunity takes place. *Adapted from Maartens, Celum, & Lewin, 2014.*

In 20% of the HIV-1-infected patients, broadly neutralising antibodies (bNAbs) appear around 3 months after infection (Walker et al., 2011). bNAbs are characterized by its exceptional potency and are directed to epitopes from many HIV-1 subtypes. However, bNAbs do not contribute to the clearance of the virus because of the arise of newly produced escape mutants (Liao et al., 2013). Recently, bNAbs are acquiring importance in the development of an effective HIV-1 preventive vaccine and for their putative usage as an alternative therapy to ART (J. Jardine

et al., 2013; For review Cohen & Caskey, 2018). A very important role plays in this context the intense effects on T cell homeostasis in the mucosa of the gastrointestinal tract due to early effects of viral replication. In this case, a massive decrease of activated $CD4^+$ T lymphocytes occurs, and it is not restored even after antiretroviral therapy (Mehandru et al., 2007). Furthermore, there is an important loss of Th17 lymphocytes as well as other T cell subsets, such as mucosal-associated invariant T cells, which are important in the defence against bacteria (Cosgrove et al., 2013; Prendergast et al., 2010). Together with the depletion of T cell subsets in the gastrointestinal tract, the prolonged production of type-I-IFN and the proinflammatory cytokines IL-6 and TNF-α secreted by activated pDCs with the subsequent recruitment of neutrophils drive the establishment of a chronic immune activation that leads to the induction of apoptosis of the enterocytes. As a consequence, translocation of microbial products into plasma takes place, with lipopolysaccharide as a potent activator of TLR4-bearing cells (Brenchley et al., 2006). Thus, the over-stimulation of the innate and adaptive immune systems ends up with the production of more proinflammatory cytokines, promoting also a chronic immune activation. At this point, it is important to note that together with $CD4^+$ T cell depletion, chronic immune activation, which starts into the gastrointestinal tract (GIT), is also a hallmark of HIV-1 infection. The residual chronic inflammation persists even after the initiation of ART and the restoration of $CD4^+$ T cell numbers and is associated with several diseases, such as liver disease (Andrade et al., 2013), cardiovascular disease (Hsue et al., 2012), cancer (Marks et al., 2013), neurological disease (Ancuta et al., 2008), and also with mortality (Kuller et al., 2008).

Immune reconstitution disease, also known as immune reconstitution inflammatory syndrome (IRIS) can appear in HIV patients shortly after the beginning of ART. It consists in an atypical immunopathological response as a result of the rapid reestablishment of pathogen-specific immune responses to pre-existing antigens in combination with a dysregulation of the immune system (reviewed in Lawn & Meintjes, 2011; Nelson, Manabe, & Lucas, 2017; Sharma & Soneja, 2011). Often, the antigens that trigger IRIS belong to pathogens that cause opportunistic infections, such as tuberculosis, cryptococcal meningitis and cytomegalovirus retinitis. An incidence of 16.1% has been reported (Muller et al., 2010). The disease causes high morbidity but low mortality (4.5%).

2.2.6. HIV-1 latency and the establishment of reservoirs

HIV-1 can infect not only activated but also resting $CD4^+$ T cells. This fact allows the virus to establish latent reservoirs which cannot be detected nor destroyed by the current available drugs since there is no expression of viral antigens. Moreover, memory $CD4^+$ T cells have a long lifespan up to several years. Studies showed that there is a proportion of one latently infected cell per every million of resting $CD4^+$ T cells (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997) and that a reservoir of 10^5-10^7 latently HIV-1-infected cells is established during the first weeks of infection in most of the individuals (Chun et al., 1997). HIV-1-Infected activated $CD4^+$ T cells can also give rise to a latent reservoir if revert back to a latent state (Pan, Baldauf, Keppler, & Fackler, 2013; Siliciano & Greene, 2011). In fact, infection of activated $CD4^+$ T cells that are reverting into a resting state provides the best conditions for the establishment of latency because of the higher expression of CCR5 in activated $CD4^+$ T lymphocytes, the proper availability of dNTP pools for reverse transcription and the immediate reduction of viral gene expression because of the inactivation of activation-dependent host transcription factors, such as the nuclear factor of activated T cells (NFAT) and NF-κB, features that are absent in resting $CD4^+$ T cells (Shan et al., 2017).

A study showed that in a non-human primate model of HIV-1 infection, the primary sites of persistence of the HIV closely related simian immunodeficiency virus (SIV) RNA and DNA are the gastrointestinal tract and lymphoid tissue (Brenchley & Douek, 2008; Estes et al., 2017; Veazey, 2019). Monocyte-derived HIV-1 infected cells are key players in the establishment of viral reservoirs. These cells can provide a reservoir to the virus in a minor proportion. As mentioned in the previous section, recent studies with penile tissue from HIV-1-infected patients showed that urethral macrophages contain integrated HIV-1 DNA, RNA, proteins and virions and that they can be considered dormant reservoirs since after reactivation they are able to produce replication-competent infectious HIV-1 particles (Ganor & Bomsel, 2011; Ganor et al., 2019).

Also, the role of monocyte-derived cells in the persistence of HIV-1 infection acquires importance because they can cross the blood-brain barrier and facilitate the infection of cells from the central nervous system (CNS), where new reservoirs of the virus can be established (Alexaki, Liu, & Wigdahl, 2008). Importantly, unlike $CD4^+$ T lymphocytes, monocyte-derived cells are resistant to the cytolytic effects of HIV-1 and can release viral particles for a prolonged period of time (Alexaki et al., 2008; Aquaro et al., 2002; Meltzer et al., 1990). In this sense, macroglial cells, which are major HIV-1 target in the brain, could work as HIV-1 reservoirs in the CNS (Schneider et al., 2015). Hematopoietic stem cells have also been suggested as a putative

reservoir of HIV-1 but several reports showed conflicting results regarding the resistance of these cells to HIV-1 infection (Alexaki et al., 2008; Josefsson et al., 2012; Nixon et al., 2013; Stanley et al., 1992; Weichold et al., 1998; J. Zhang, Scadden, & Crumpacker, 2007). Regarding the mechanisms of latency establishment, several studies showed that repressive chromatin states play an important role (Blazkova et al., 2009; Kauder, Bosque, Lindqvist, Planelles, & Verdin, 2009; Pion et al., 2003; Trejbalová et al., 2016). Among them we find obstructive nucleosome positioning, DNA methylation, and posttranscriptional modifications of histones and other proteins. Other studies showed alternative mechanisms contributing to the establishment of a latent reservoir, such as low levels of viral Tat protein and host transcription factors, defects in RNA splicing and export or transcriptional interference.

2.2.7. Acquired Immunodeficiency Syndrome

During the course of chronic HIV-1 infection the exhausted immune system is outpaced by a series of pathogens of different etiology, which are the cause of well-defined HIV-related opportunistic infections (Deeks, Overbaugh, Phillips, & Buchbinder, 2015; Moir, Chun, & Fauci, 2011; Yasuoka, 2010). Ultimately, HIV infection-derived opportunistic infections are the cause of the dead of the patient. It is considered that a patient gets into AIDS either when the number of $CD4^+$ T cells in blood is lower than 200/ml (Doitsh & Greene, 2016), or when the patient presents one of some clinical manifestations included in AIDS definition by World Health Organization (WHO) in 1987. There are three categories related to the symptoms that a patient experience within HIV infection (Categories A, B and C), independently of the number of $CD4^+$ T lymphocytes in blood **(Table 2)**. Category A includes the patients that present primary infection or that are asymptomatic. Category B includes the patients that present symptoms that are not within C category but that are related to HIV infection. Examples of B class clinical manifestations are pelvic inflammatory disease (PID) (Korn, 1998), immune thrombocytopenic purpura (Yospur, Sun, Figueroa, & Niihara, 1996), or mild fever during more than one month. Examples of C class clinical manifestations can be caused by bacteria: tuberculosis, septicaemia caused by *Salmonella*; of viral origin: cytomegalovirus infection or Herpes simplex virus (HSV) 1 or 2 outbreak; of fungal origin: Aspergillosis, candidiasis, histoplasmosis; caused by protozoa: pneumonia, neurological toxoplasmosis, isosporiasis (Yasuoka, 2010); processes directly related to HIV: wasting syndrome (Emerole, Pokrovskaya, & Pilipenko, 2016), progressive multifocal leukoencephalopathy (Cinque, Koralnik, Gerevini, Miro, & Price, 2009), HIV-related dementia

(Eggers et al., 2017; Smail & Brew, 2018), or tumoral processes: Kaposi´s sarcoma, Burkitt lymphoma or cervical cancer (Ji & Lu, 2017).

$CD4^+$ T LYMPHOCYTES	A	B	C
> 500 CELL/ML	A1	B1	C1
200-500 CELL/ML	A2	B2	C2
< 200 CELL/ML	A3	B3	C3

Table 2: Categories of HIV-1-infected patients. Category of HIV-1-infected patients (A, B and C) in relation to the number of CD CD4 T lymphocytes in blood$^+$. In orange AIDS. Category A includes patients during the early onset of the infection and also patients that are asymptomatic at any time during the infection. Category B includes patients that are not in category C but present other clinical manifestations related to HIV-1 infection. Examples of category B patients are the ones who suffer PID or immune thrombocytopenic purpura. Category C patients include the ones who suffer typical late AIDS clinical manifestations such as opportunistic infections or HIV-1 related tumors.

2.2.8. Antiretroviral therapy against HIV-1 infection

Since the appearance of the first HIV-1 specific antiviral drug, Zidovudine (ZDV); also known as Azidothymidine (AZT)), a nucleoside-analog reverse transcriptase inhibitor (NRTI) in 1987, which was given as a monotherapy or in combination of other early reverse transcriptase inhibitors (zidovudine, didanosine, zalcitabine, stavudine, and lamivudine), leading the selection of clones that were multi-drug resistant (Iversen et al., 1996; Schmit et al., 1996), a considerable evolution in the standard HIV-1 infection care has happened. Nowadays, the most effective HIV-1 treatment includes a combination of up to three drugs in a cocktail, known as highly active antiretroviral therapy (HAART) (Henkel, 1999), that targets not only reverse transcriptase action but different essential steps on HIV-1 replication cycle (for a complete review (Arts & Hazuda, 2012) **(Figure 11)**. Antiretroviral drugs are distributed into six distinct classes: (1) nucleoside-analog reverse transcriptase inhibitors (NRTIs), (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (3) integrase inhibitors (INIs or INSTIs), (4) protease inhibitors (PIs), (5) fusion inhibitors, and (6) coreceptor antagonists (CCR5 antagonists). Before 1996, the treatment strategy consisted in prophylaxis against common opportunistic pathogens and the control of

HIV-1 related illnesses that could appear, besides the administration of an antiretroviral drug. As mentioned in the preceding lines, the first antiretroviral drugs were administrated as a monotherapy, but the advent of drug resistance due to the selection of clones obligated scientist to find new solutions. The combination of drugs attacking different steps of the replication cycle given as a cocktail eliminated, or at least delayed considerably the resistance issue (Henkel, 1999).

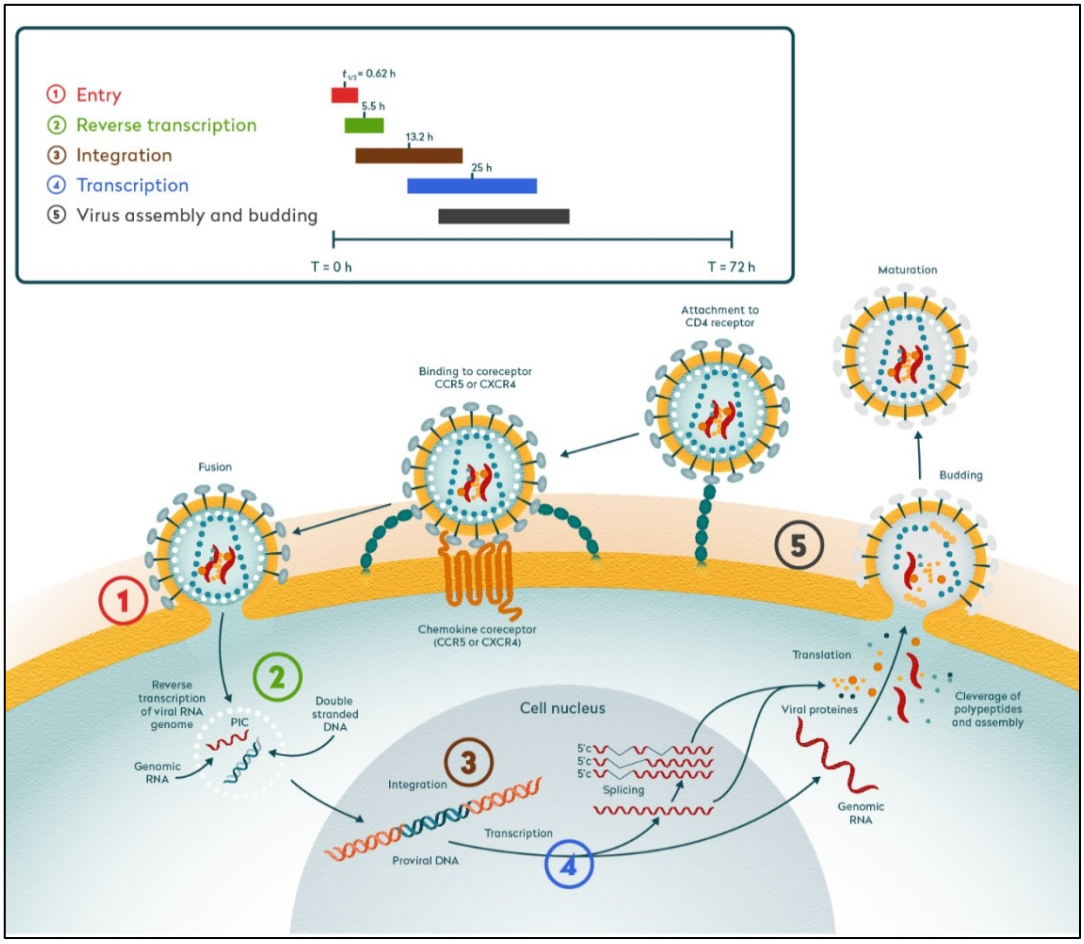

Figure 11: Different steps in HIV-1 replication that are potential targets for antiretroviral drugs. Scheme of HIV-1 replication cycle indicating with numbers (1-5) the steps to target with antiretroviral drugs. Namely, attachment and fusion (entry), reverse transcription, DNA integration, transcription, virus assembly and budding. In the box is showed a time frame for antiviral drug action during a single-cycle HIV-1 replication assay. If a step-specific drug is added after the step has took place, it will result in a lack of inhibition.

PIC, pre-integration complex; CCR5, CC-chemokine receptor 5; CXCR4, CXC-chemokine receptor 4

HAART was crucial in the reduction of morbidity and mortality associated to HIV-1 infection and AIDS (Collier et al., 1996; D'Aquila et al., 1996; Staszewski et al., 1996). Combinational therapy against at least two different targets reduces efficiently plasma viral load under the limit of detection of commercial assays (<50 copies/ml) allowing the reconstitution of the immune system taking $CD4^+$ T lymphocytes as a measure (Autran et al., 1997; Komanduri et al., 1998; Lederman et al., 1998) **(Figure 12)**. However, the effect of therapy can be reduced or impaired if the patient shows no adherence to the treatment, for poor drug tolerability or interaction with other antiretroviral or non-antiretroviral drugs that can cause a reduction of the optimal drug levels.

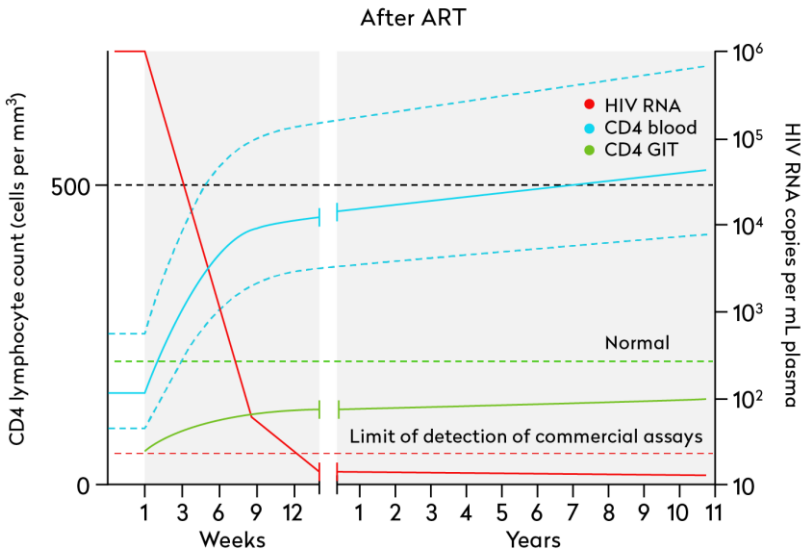

Figure 12: HIV-1 load and $CD4^+$ T cells dynamics in ART-treated infected patients. In ART-treated HIV-infected patients, HIV load decreases rapidly under the limit of detection of commercial assays during the first weeks after the treatment starts. CD4 T cell numbers recover progressively during the following months and years of infection without reaching normal pre-infection levels. Recovery of CD4 T cells in the gastrointestinal tract is reduced compared to recovery of CD4 T cells in blood. *Adapted from Maartens et al., 2014.*

ART, antiretroviral therapy; GIT, gastrointestinal tract.

Nowadays, patients can take the combination of antiretroviral drugs in commercial fixed-dose combinations, which consist of a combination of two, three or four drugs into one single pill that is taken daily, thus, facilitating the adherence of the patient to the medication and improving effectiveness over the long-term. Examples of fixed-dose combinations are Dutrebis,

which combines lamivudine and rageltavir, or Triumeq, which combines abacavir, dolutegravir and lamivudine (Bangalore, Kamalakkannan, Parkar, & Messerli, 2007; Bangsberg, Kroetz, & Deeks, 2007) **(Table 3)**.

The first step on HIV-1 replication cycle that is targeted by antiretroviral drugs is viral entry. Here, fusion inhibitors, such as enfuvirtide (T-20) have been developed in order to block virion penetration within the target cell (Lalezari et al., 2003). Targeting the coreceptor CCR5 with CCR5 antagonists, such as maraviroc (MVC) is another way to stop the replication cycle at this point ("Maraviroc reduces viral load in naive patients at 48 weeks.," 2007). Recently, a new drug that blocks the entrance of the virus has been approved by the U.S. food and drug administration (FDA). Ibalizumab is a "post-attachment inhibitor", constituted by a non-immunosuppressive humanized monoclonal antibody that binds CD4 and blocks HIV attachment to the coreceptors CCR5 or CXCR4 (Jacobson et al., 2009). The following event that is targeted is reverse transcription. NRTIs and NNRTIs block the function of reverse transcriptase. NRTIs block the enzyme by joining the catalytic domain and NNRTIs bind to a non-catalytic allosteric pocket on reverse transcriptase. When the enzyme is blocked there is no generation of DNA and HIV-1 replication cycle cannot be completed. There are plenty of reverse transcriptase inhibitors available nowadays, being abacavir (ABC) and tenofovir (TDF), which was were by the Czech chemist Antonín Holý in the Institute of Organic Chemistry and Biochemistry (IOCB) in Prague, examples of NRTIs (Ustianowski & Arends, 2015), and efavirenz (EFV) and nevirapine (NVP) examples of NNRTIs (De Clercq, 1998). The third step of the replication cycle that can be blocked is the integration of the viral DNA into the host DNA. INIs block the viral integrase which oversees DNA integration. Specifically, dolutegravir (DTG) and raltegravir (RAL) block the strand transfer step within integration (Markowitz et al., 2007). PIs are a series of drugs that target the viral protease that is in charge of the cleavage of newly synthetized viral proteins, a process that takes place inside the immature virion, after the budding. By targeting the viral protease immature virions cannot mature and become functional. Examples of PIs are atazanavir (ATV) and tipranavir (TPV). Cobicistat (COBI) and ritonavir (RTV) (Lv, Chu, & Wang, 2015). are pharmacokinetic enhancers which inhibit liver enzymes that metabolizes the other antiretroviral drugs, allowing a prolonged action span of these (Lianhong Xu et al., 2010; Zeldin & Petruschke, 2004).

Generic name	Brand name	FDA aproval date
Nucleoside reverse transcriptase inhibitors (NRTIs)		
abacavir (ABC)	Ziagen	1998
emtricitabine (FTC)	Emtriva	2003
lamivudine (3TC)	Epivir	1995
tenofovir (TDF)	Viread	2001
zidovudine (AZT)	Retrovir	1987
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)		
doravirine (DOR)	Pifeltro	2018
efavirenz (EFV)	Sustiva	1998
etravirine (ETR)	Intelence	2008
nevirapine (NVP)	Viramune	1996/2011
rilpivirine (RPV)	Edurant	2011
Protease Inhibitors (PIs)		
atazanavir (ATV)	Reyataz	2003
darunavir (DRV)	Prezista	2006
fosamprenavir (FPV)	Lexiva	2003
ritonavir (RTV)*	Norvir	1996
saquinavir (SQV)	Invirase	1995
tipranavir (TPV)	Aptivus	2005
Fusion inhibitors (FIs)		
enfuvirtide (T-20)	Fuzeon	2003
CCR5 antagonists		
maraviroc (MVC)	Selzentry	2007
Integrase inhibitors (INIs)		
dolutegravir (DTG)	Tivicay	2013
raltegravir (RAL)	Isentress	2007/2017
Post-Attachment inhibitors		
ivalizumab-uiyk (IBA)	Trogarzo	2018
Pharmacokinetic Enhancers		
cobicistat (COBI)	Tybost	2014

Table 3: List of HIV drugs recommended for the treatment of HIV infection in the United States by April, 2019. All the drugs on the list have been approved by the U.S. Food and Drug Administration (FDA). Even though it was developed as a protease inhibitor, ritonavir is widely

used as a pharmacokinetic enhancer in combination with other antiretroviral drugs. *Source "aidsinfo.nih.gov".*

2.2.9. Perspectives for a cure

HIV-1 is considered nowadays a life-long chronic infection due to the effectivity of the combined antiretroviral therapy. The major problem that scientists face is the consecution of a sterilizing cure by means of the selective elimination of the latently infected cells that allow the virus to re-emerge in the case of interruption of ART. Until recently, only two individuals, known as the "berlin and London patients", had been officially cured from HIV-1 infection, by exhibiting a prolonged post-treatment control of HIV viral load after ART was interrupted. Notably, the case of Timothy Ray Brown, the Berlin patient, is an especial issue because of the nature of the diagnostic and the treatment followed to get to the cure. Apart from HIV-1 infection, he was diagnosed with acute myeloid leukemia. Two hematopoietic stem cell transplant (HSCT) from a homozygous CCR5 delta32 donor were done in 2007 and 2008 after eradication of his own immune system by chemotherapy and irradiation. The same day of the first HSCT the patient stopped taking ART and three months later HIV viral load decreased dramatically below undetectable levels at the same time that $CD4^+$ T cell number increased significantly (Hutter et al., 2009). The Berlin patient was still free of HIV RNA up to his death in 2021 due to myeloid leukemia. On March 2019, a second HIV-I-infected individual, known as the London patient ,who got HSCT in the same terms of the berlin patient three years ago after being diagnosed with Hodgkin´s lymphoma has been reported as the second person to be cured of HIV-1 infection (Gupta et al., 2019). Moreover, a third case of a putative cured individual known as the Düsseldorf patient may be confirmed soon. Even the good news that suppose being able to reach a sterilizing cure, the case of these patients cannot be compared to the majority of the HIV-1-infected population, which is living under "normal" conditions with the aid of ART. The severity and risk of taking a HSCT, which has a mortality rate of 50%, pushes scientists to find alternative solutions to find either a sterilizing cure, in which patients are cleared completely from the virus, or a functional cure, in which patients are able to control the viral load and function.

The major problem when facing the eradication of the virus from the organism is to kill the reservoirs of latently infected cells, which repopulate the virus after the patient interrupts ART. Most efforts are focused in the so called "shock and kill" strategy (reviewed in Pitman, Lau, McMahon, & Lewin, 2018; Schwartz et al., 2017), which consists in the usage of latency-

reversing agents (LRAs) in order to make the latently infected cell to produce viral proteins, thus, being able to be detected and eliminated by the immune system or to lead directly to virus-mediated cell death **(Figure 13)**. Numerous LRAs, such as, histone deacetylase inhibitors (HDACis) (Blazkova et al., 2009; Elliott et al., 2014; Richard Brad Jones et al., 2014; Pace et al., 2016; Rasmussen et al., 2014; Sogaard et al., 2015), Disulfiram (Elliott et al., 2015), or TLR agonists (Tsai et al., 2017; Vibholm et al., 2017) have been tested, but so far, although they transitionally reduced the number of infected cells, they failed in eradicating the HIV-1 reservoir.

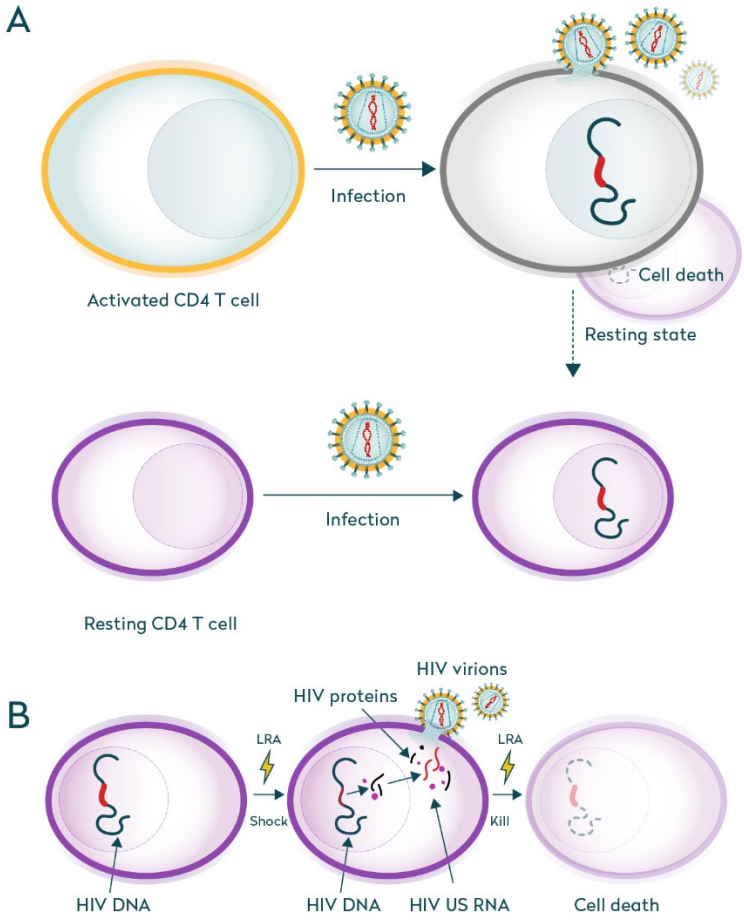

Figure 13: Latency and reactivation through "shock and kill". (A) Latency can be established when HIV infects a cell that is undergoing the process to a resting state or when HIV infects a resting CD4 T cell directly. (B) The "shock" consists in the exposition to LRAs and the killing is mediated by direct immune action over the infected cell in combination with the ART. *Adapted from Maartens et al., 2014.*

LRA, latency-reversing agents; ART, antiretroviral therapy; HIV US RNA, HIV unspliced RNA.

Only a study with macaques led to sustained remission of SIV when treated with the TLR7 agonists vesatolimod and GS986 (Lim et al., 2018). These results open the door to clinical trials of these new TLR7 agonists on HIV-I-infected humans. However, there is still the need for a search of less toxic, more potent and more specific LRAs. Another strategy to find the cure of HIV may be the usage of gene editing. The aim would be to modify the sequences of CCR5 coreceptor on T cells and stem cells or even of viral proteins. Clinical trials with zinc finger nucleases (Tebas et al., 2014) and CRISPR/Cas-9 (Z. Liu et al., 2017) have been performed and others are being held already in phase 1 and 2. The main issue with the edition of HIV genome is the requirement of multiple targets in order to avoid resistance. Moreover, an enhanced delivery method to target only the infected cells will be necessary.

	Rationale	Key findings	Future directions
Strategies targeting the viral replication cycle			
Bone marrow transplantation	Transplanted cells from a CCR5Δ32 donor are resistant to HIV infection; transplanted cells can potentially eliminate infected cells in the recipient via graft versus host disease	Transplantation with CCR5Δ32 donor cells has cured only one individual,[1] and is associated with high rates of mortality;[46] transplantation of wild-type CCR5 donor cells reduced HIV persistence on ART and delayed viral rebound once ART was stopped[7]	Large observational cohort studies of transplant recipients; optimise transplantation with modified and wild-type donor cells in non-human primates
Gene editing	Alter host proteins such as CCR5 so that T cells are resistant to HIV infection	Transfusion of genetically modified T cells that do not express CCR5 is safe, and modified cells persist in vivo but in low numbers;[48] transfusion of modified CD4 T cells did not delay viral rebound once ART was stopped[48]	Enhance the frequency and tissue penetration of gene-modified cells through gene editing of stem cells, novel conditioning regimens, and newer gene-editing technologies;[49] use gene-editing strategies to target HIV itself and other host proteins such as HIV restriction factors
LRAs	Increase transcription of HIV to induce virus-mediated or immune-mediated cytolysis	Can increase cell-associated HIV RNA, and in some cases plasma HIV RNA, but have so far not been shown to decrease the number of infected cells in human studies;[26–28,50,51] although safe, some concerning adverse effects of histone deacetylase inhibitors have been reported on host gene transcription[28] and T-cell function[52,53]	Combine LRAs with drugs that can enhance clearance of infected cells; increase potency and specificity, reduce toxicity, and enhance delivery of LRAs to tissue sites
HIV-specific immune enhancement strategy			
T-cell vaccines	Increase HIV-specific T-cell immunity to maintain long-term remission and kill latently infected cells	Number and function of HIV-specific T cells increase after vaccination; the combination of T-cell vaccines with the LRA romidepsin led to enhanced virus control after ART cessation[54] but, in the absence of a placebo group, interpretation of these findings is difficult[55]	Combine T-cell vaccines with new adjuvants (eg, toll-like receptor 7 agonists),[56] new antigens (eg, mosaic proteins), new delivery systems (eg, electroporation), and new vectors (eg, cytomegalovirus vector),[57] or develop an RNA vaccine
bNAbs	Administer antibodies that target many HIV strains and can bind and clear free virions and potentially infected cells	Administration of a single bNAb to HIV-infected individuals on ART resulted in a delay to viral rebound after ART cessation, compared with historical controls;[58,59] rebound virus was often resistant to the bNAb;[58,59] whether bNAbs eliminate infected cells in vivo or just neutralise rebounded virus remains unclear	Assess combinations of bNAbs to reduce the emergence of resistance, develop new longer-acting antibodies, develop bispecific and trispecific bNAbs, and combine bNABs with LRAs
CAR T cells	Allow strong antigen-specific T-cell responses that are independent of MHC restriction	CAR T cells with a bNAb single-chain variable fragment in the CAR extracellular domain can lyse HIV-infected cells ex vivo[60]	Minimise toxicity from cytokine release
Immune modulation strategy			
Immune-checkpoint blockers	Enhance the HIV-specific functional immune response and potentially reverse HIV latency	The safety profile of immune checkpoint blockers is of some concern, particularly with regards to immune-related adverse events; case reports show these blockers can cause perturbation of the HIV reservoir, but very few individuals have been assessed[61,62]	Conduct large observational cohort studies of individuals with HIV and cancer who receive immune checkpoint blockers; conduct dose escalation studies in individuals with HIV but without cancer to assess treatment safety; investigate the use of immune checkpoint blockers alone and in combination in animal models
Vedolizumab	Reduce trafficking of highly susceptible CD4 T cells to the gut HIV reservoir	Prolonged viraemic control after analytical treatment interruption in a non-human primate model[5]	Results of human clinical trials are awaited
Interleukin 15 superagonist	Improve trafficking of CD8 T cells into lymphoid-tissue B-cell follicle reservoirs	Increased CD8 T-cell CXCR5 expression and infiltration into B-cell follicles of lymph nodes in macaques[63]	Results of human clinical trials are awaited
Sirolimus	Reduce HIV-associated inflammation, thereby improving the HIV-specific immune response	Associated with reduced HIV DNA concentrations in a renal transplant cohort study[64]	Results of human clinical trials are awaited

CCR5=C-C chemokine receptor type 5. ART=antiretroviral therapy. LRA=latency reversal agent. bNAb=broadly neutralising antibody. CAR=chimeric antigen receptor.

reservoir after ART recommencement[47] | transmission has not been reported to date

ART=antiretroviral therapy. QVOA=quantitative viral outgrowth assay. TILDA=tat/rev induced limiting dilution assay. LTR=long terminal repeat.

Table 4: Summary of the recent/current on-going strategies for an HIV cure that are on clinical trials. *Adapted from Pitman et al., 2018*

Another idea that scientists are exploring is to inhibit the antiapoptotic protein B cell lymphoma 2 (BCL2) which is found in high concentrations in resting $CD4^+$ T cells compared to activated $CD4^+$ T cells. The proapoptotic inhibitor of BCL2 Venetoclax, used in combination with LRAs showed a decrease on HIV DNA suggesting the selective depletion of infected cells (Cummins et al., 2016). This strategy received the name of "prime, shock and kill" for obvious reasons. Finally, silencing has also been proposed as a "block and lock" strategy. The addition of an HIV Tat inhibitors (Kessing et al., 2017; Mousseau et al., 2012) or RNAi (Ahlenstiel et al., 2015; Centlivre et al., 2013) in order to silence the provirus transcription have been studied with *a priori* positive results, but the selective delivery of the silencing agents into infected cells must be improved. Another area of on-going investigation is the study of the administration of an HIV therapeutic vaccine combined with LRAs and other adjuvants in order to direct the $CD8^+$ T cell-mediated elimination of infected cells. Clinical trials showed relative success (Sneller et al., 2017) but the problem of this method is the appearance of escape mutants. In this sense, broader cytotoxic $CD8^+$ T cell-mediated responses are needed in order to embrace the range of HLA alleles (Deng et al., 2015; Hancock et al., 2015). Broadly neutralizing antibodies are able to neutralize a wide range of HIV-1 strains not only at a free virion level but also can mediate the depletion of infected cells expressing viral antigen on its surface by the action of phagocytes, NK cells and $CD8^+$ T cells (Bruel et al., 2016; Lu et al., 2016; Nishimura et al., 2017; Schoofs et al., 2016). Clinical trials showed that the administration of the bNAbs 3BNC117 and VRC01 to HIV-1 infected patients taking ART delayed viral rebound after ART interruption. Even though, with enough time, these antibodies can generate escape mutants (Bar et al., 2016; Scheid et al., 2016). Importantly, studies with non-human primates revealed that bNAbs are capable of clearing SIV containing HIV env (SHIV) from infected neonates (Hessell et al., 2016). Similar studies in humans are being assessed. bNAbs have been used in combination with bispecific and trispecific antibody-based molecules and showed a big potency in neutralizing HIV in vitro (Y. Huang et al., 2016; Ling Xu et al., 2017). Other studies *in vitro* using engineered proteins called dual-affinity retargeting molecules (DART), which are formed by heterodimers comprising the variable regions of two monoclonal antibodies with specificity against HIV and CD3 are able to direct cytolytic lymphocytes to infected cells and facilitate its clearance (Sloan et al., 2015). Clinical trials are being done with chimeric antigen receptor (CAR) expressed on T cells. CARs consist on an extracellular domain derived from a monoclonal antibody or a cell surface receptor or ligand, attached through a transmembrane domain to a CD3 signalling ζ chain. CAR-expressing T cells are able to lyse HIV-infected cells *in vitro* and *in vivo* (Hale et al., 2017; B. Liu et al., 2016). The last group of studies are based on immune modulation in order to achieve a functional cure. Checkpoint markers are surface molecules which trigger regulatory pathways to reduce T-cell

activity. Examples of these molecules are PD-1 or cytotoxic T-lymphocyte antigen 4 (CTLA-4). The expression checkpoint markers are upregulated in HIV infection (Chomont et al., 2009; Fromentin et al., 2016; McGary et al., 2017). The usage of mAbs against these molecules has been shown to intensify HIV-specific T cell response *ex vivo* and they could act also as LRAs (Chew et al., 2016; Wykes & Lewin, 2018). The first observational trials in humans resulted in immune-related toxicity (Gay et al., 2017) and currently several independent studies (Guihot et al., 2018; Wightman et al., 2015) and clinical trials are taking place in order to find safer immune-checkpoint blockers with higher HIV-specific enhancement of the immune function. Modifying T cell trafficking to tissues is another potential cure strategy. The administration of a mAb against α4β7, an integrin that favors gut-homing, to SIV-infected, ART-suppressed macaques resulted in the maintenance of aviremia for 2 years after treatment interruption due to enhanced trafficking to gut and immune complexes formation (Byrareddy et al., 2016). Alternatively, increasing the expression of the chemokine receptor CCR5 on cytotoxic T cells enhanced the trafficking to the lymph nodes, where latent reservoirs can persist (Fukazawa et al., 2015). Clinical trials of both trafficking-modifying strategies are currently being held. The level of inflammation during the chronic phase of HIV infection has been for long correlated with progression to immune exhaustion and finally progression to AIDS (Khoury et al., 2017). Researchers are exploring the usage of anti-inflammatory drugs in order to reduce HIV persistence. By targeting the serine/threonine kinase mTOR with an inhibitor, a reduction in HIV DNA has been accomplished after a kidney transplantation (Stock et al., 2014). Another obvious way to reduce the inflammation is by blocking the effects of IFN I. In studies with humanized mice, the administration of an antibody against IFN receptor reduced immune activation, enhanced T cell HIV-specific responses an caused a significant delay in viral rebound by reducing viral reservoirs in lymphoid tissue (Cheng et al., 2017).

Last years have seen the arousal of studies involving the so called post-treatment controllers (PTCs) as a model of a functional cure, in which HIV-1-infected patients are able achieve viral control after ART interruption in a similar way as HIV-1 infected elite controllers (ECs) do (Cockerham, Hatano, & Deeks, 2016; Davenport et al., 2019; de Bree & Sanders, 2019; Etemad, Esmaeilzadeh, & Li, 2019; Goulder & Deeks, 2018; Maenza et al., 2015; Martin & Frater, 2018; Sáez-Cirión et al., 2013). The difference between ECs and PTCs is that the former group can spontaneously maintain a low viral load without the use of ART, whereas PTCs maintain low levels of virus after treatment interruption. There have been several PTCs cohort studies in last years (Etemad et al., 2019). Interestingly, evidence has been found of several phenotypic differences between PTCs and ECs. For instance, certain human leukocyte antigen (HLA) alleles

have been associated with protective effects in ECs whereas PTC do not appear to possess these alleles. In fact, the VISCONTI study reported a correlation between the presences of the aforementioned protective alleles in PTCs with a less favorable clinical outcome (Sáez-Cirión et al., 2013). Also, cytotoxic lymphocyte (CTL) responses have been found to be stronger in ECs compared to PTCs among other differences. Altogether, the analysis of these studies have leaded to the conclusion that an early ART initiation during HIV-1 acute phase of infection significantly increases the chances of achieving post-treatment control allowing the adaptive immune response to prevent viral rebound and control infection (Etemad et al., 2019). Upcoming investigations with PTC cohorts are necessary to uncover the mechanism of post-treatment control and facilitate the path towards a functional cure of HIV-1 infection.

2.3. PDC activation in HIV-1 infection

Even though pDCs are not considered a reservoir of HIV-1, they can interact and be infected by HIV-1 since they express HIV-1 receptor CD4 and the co-receptors CCR5 and CXCR4 (O'Brien, Manches, & Bhardwaj, 2013). After the virion is internalized *via* dynamin-dependent endocytosis, HIV-1 sensing induces the production of large amount of IFN-I but low amounts of proinflammatory cytokines (TNF-α and IL-6), thus, leading the cell to become an interferon-producing cell rather than an APC (Beignon et al., 2005; Haupt et al., 2008; McKenna, Beignon, & Bhardwaj, 2005; O'Brien et al., 2011; Pritschet et al., 2012). The sensing of HIV and subsequent IFN-I production is mediated via TLR7 and IRF7 activation rather than TLR9, given that TLR7 inhibitors are much more potent than TLR9 in abolishing IFN-I production by HIV-exposed pDCs (Aiello, Giannessi, Percario, & Affabris, 2018; Pritschet et al., 2012). The intensity of IFN-I response is determined by the affinity of gp120 to CD4 and not by CXCR4 or CCR5 (Haupt et al., 2008). Even though upstream events that lead to IFN-I production are still not clear, it is known that HIV endocytosis and endosomal acidification but not fusion or viral replication are necessary (Beignon et al., 2005). Importantly, cell-free HIV-1 virions are weak inducers of IFN-I compared to other viruses (Beignon et al., 2005; Lo et al., 2012). In this regard, it was reported that pDCs are markedly resistant to HIV-1 infection because of the high expression of the restriction factor SAMHD1, an enzyme with phosphohydrolase activity which depletes the dNTP pool necessary for reverse transcription and replication of the virus (Bloch et al., 2014). On the other hand, it was shown that HIV-1-infected $CD4^+$ T lymphocytes are good inducers of type-I-interferon secretion by pDCs (Schmidt, Ashlock, Foster, Fujimura, & Levy, 2005). The mechanisms of these events are still poorly understood. Contrarily to mDCs, pDCs have the

notorious ability of not becoming refractory after a subsequent activation. This may be due to the requirement of the positive autocrine feedback through IFNAR receptor to acquire full functionality and because of the prolonged localization of the virus in early endosomes (O'Brien et al., 2016).

Notably, studies on the context of HIV infection have shown that IFN-α triggers the expression of the pro-apoptotic molecule TNF-related apoptosis-inducing ligand (TRAIL), turning pDCs into IFN-producer Killer pDCs (IKpDCs). IKpDCs are able to induce apoptosis of HIV-infected and non-infected $CD4^+$ T cells indistinctly through the activation of DR-5 signalling pathway. These reports suggest a direct mechanism of pDCs-mediated of $CD4^+$ T cells of HIV-infected patients, which is a hallmark of HIV infection (Gougeon & Herbeuval, 2012; Hardy, Graham, Shearer, & Herbeuval, 2007). Remarkably, studies in humanized mice showed that when pDCs were depleted before or during the acute phase of HIV-1 infection, HIV-1 replicated to high levels in all tissues and there was no signature of IFN-I or ISGs induction. At the same time, $CD4^+$ T cell depletion was significantly reduced compared mice with normal pDC numbers. Therefore, at least on these mice model of humanized mice, pDCs were the major source of IFN-1 during acute HIV-1 infection. IFN-1 levels directly correlated with viral load and inversely correlated with $CD4^+$ T cell depletion (Cheng, Ma, Li, & Su, 2018; Cheng et al., 2017; G. Li et al., 2014; Su, 2019).

2.4. The role of pDC in the chronic phase of HIV-1 infection

The chronic phase of HIV-1 infection is characterized by an aberrant immune response in which $CD4^+$ and $CD8^+$ T lymphocytes overexpress HLA-DR, CD38, ki67 and inhibitory molecules such as CTLA-4 and PD-1 (Day et al., 2006; Kaufmann et al., 2007; Trautmann et al., 2006). This aberrant response is defined as well by T cell exhaustion and increased apoptosis levels and is persistent even under ART. There is a better correlation between disease progression to AIDS (with the subsequent apparition of co-morbidities) with the abnormal response of the immune system than with viremia (Baker & Duprez, 2010; El-Sadr et al., 2006; Ho et al., 2010; Lekakis & Ikonomidis, 2010; Lichtenstein et al., 2010). Importantly, disease progression to AIDS and the appearance of associated co-morbidities correlate with IFN-1 levels in plasma. Studies with non-human primates showed that non-pathogenic SIV-infected natural hosts, such as sooty mangabey (SM) and african green monkey (AGM), exhibit high viral load, low immune activation and poor type-I-IFN levels compared to pathogenic SIV-infected non-human primates, such as

rhesus macaque (RM), which display high immune activation and high IFN-I levels in plasma (Bosinger et al., 2009; Jacquelin et al., 2009). In HIV-infected patients has been observed a chronic production of the IFN-α subtype IFN-α2b (C Lehmann et al., 2009). Circulating pDC numbers decline is a feature of HIV-I infection and correlates T $CD4^+$ depletion and high viral load (Donaghy et al., 2001). It has been found that during HIV-I infection circulating pDCs in blood express higher levels of the lymph-node homing markers CCR7 and CD62L and in fact, a higher migration of pDCs to lymphoid tissue compared to non-infected controls has been observed (Clara Lehmann et al., 2010). Therefore, the decrease of circulating pDC levels may be due to a redistribution to the lymphoid compartment rather than a depletion due to apoptosis and the cytopathic effect of HIV, at least partly. Contrarily to circulating pDCs, which show exhaustion and hypoactivation, therefore, producing low levels of IFN-I during chronic infection, pDCs allocated in the lymph nodes remain activated and produce high quantities of IFN-1 compared to non-infected healthy controls (Clara Lehmann et al., 2008). A study with HIV-1-infected patients and a humanized mice model has shown that the loss of CD34+CD38- early Hematopoietic stem cells (HSC) during chronic HIV-1 infection is dependent on pDCs presence. In the same work, depletion of pDCs prevented CD34+CD38- early HSC loss (G. Li et al., 2017). In the case of GALT (gut-associated lymphoid tissue), it is thought that the persistence in time of pDC activation may be caused by the loss of integrity of the tissue after HIV-1 infection and the subsequent translocation of bacterial particles, which would maintain the proinflammatory state (V. C. Lombardi & Khaiboullina, 2014). Moreover, during the course of infection blood circulating pDCs have a different behaviour following the stimulus with TLR7 and TLR9 agonists. During acute infection pDC are extremely sensitive and hyperreactive to *ex vivo* stimuli (Sabado et al., 2010). On the other hand, during chronic infection pDCs secrete a reduced amount of IFN-1 after stimulation with several TLR7 and TLR9 agonists (Kaushik et al., 2013; Tilton et al., 2008). In the chronic phase of HIV-infection, pDC exhaustion correlates with the level of expression of TIM-3 on its membrane, which parallelly correlates negatively with its IFN-producing capacity (J. A. Schwartz et al., 2017). Because like HIV, HCV is transmitted through direct contact with the blood of an infected person many HIV-infected patients are also infected with HCV. According to WHO, it is estimated that HIV-HCV coinfected patients comprise 2-15% of the total HIV-infected population. As for 2017, 2.75 million people were estimated to be HIV-HCV conifected globally, 1.3 million of which were people who inject drugs . In my two secondary publications, the interaction between pDCs and HCV is explored (Aouar et al., 2016; Janovec et al., 2018).

2.5. PDCs in the context of HCV infection

HCV is an enveloped positive-sense single-stranded RNA virus that belongs to the *Flaviviridae* family and it is the causing agent of a chronic infection affecting over 70 million people as of 2020, with approximately 2 million new infections occurring annually ("Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study.," 2017). Chronic Hepatitis C infection is associated with advanced liver disease and is a main cause of hepatocellular carcinoma (Goto, Roca Suarez, Wrensch, Baumert, & Lupberger, 2020; A. Lombardi & Mondelli, 2019; Rabaan et al., 2020). The development of interferon-free direct-acting antivirals (DAAs) has supposed a revolution in the management of HCV infection (Martinello, Hajarizadeh, Grebely, Dore, & Matthews, 2018). DAAs are divided in three classes, each one targeting a different HCV non-structural protein: NS3/4A protease inhibitors (Telaprevir, Boceprevir), NS5A polymerase inhibitors (e.g. Elbasvir, Ledipasvir) and NS5B polymerase inhibitors (e.g. Sofosbuvir, Dasabuvir). Treatment with a combination of two or more HCV DAAs from different classes for a relative short time (8-12 weeks) achieves very high sustained virological response (SVR) (>95%), an indicator based on the undetectability of HCV RNA levels after treatment (Götte & Feld, 2016; Michael P Manns et al., 2017).

Only a minority of patients can spontaneously clear the virus during acute infection (15-25%) (Grebely et al., 2014; Micallef, Kaldor, & Dore, 2006). The elimination of the virus during acute infection correlates with an early strong innate immune response characterized by a strong induction of ISGs and also correlates with a delayed adaptive immune response (Chigbu, Loonawat, Sehgal, Patel, & Jain, 2019; Heim, 2013; Heim & Thimme, 2014). Interestingly, treatment with IFN-α leaded to the elimination of HCV in more than 50% of cases of chronic infection (Fried et al., 2002; M P Manns et al., 2001). Being pDCs the major IFN-I producers they are thought to play an important role in the clearance of HCV in the early phase of infection. Following this concept (hypotheses), several investigations aimed to shed light on this issue.

Unlike pDCs exposed to cell-free virions, pDCs exposed through cell-to-cell contact to HCV-infected hepatocytes have been shown to produce large amounts of IFN-I *via* TLR-7 signalling (Coléon, Assil, & Dreux, 2019). This behaviour suggested that HCV free-virions are able to evade pDC-mediated immune response after direct contact. Further investigations showed that, in fact, HCV is a weak inducer of pDC-mediated IFN-α production when compared to Influenza or Human Herpes virus type-I and that at least one of the mechanisms by which HCV is able to avoid ISGs induction is mediated by the aforementioned RRs (Gondois-Rey et al., 2009). In this sense, Florentin et al., showed that HCV glycoprotein E2 acts as an inhibitor of the IFN response

when ligated to BDCA-2 surface RR on pDCs (Florentin et al., 2012). Another investigation with the pDC cell line GEN2.2 showed that the extracellular HCV core protein, which is found in circulation during chronic infection, supressed IFN-I and IFN-III production in response to TLR and the HCV PAMP agonist of RIG-I. The suppression of IFN response by the core protein correlated with downmodulation of the normally abundant in pDCs IRF-7 transcription factor, which drives the IFN response and is itself an ISG (Stone et al., 2014). Therefore, an alternative mechanism by which HCV controls the antiviral innate response was elucidated. Interestingly, two genome-wide association studies (GWAS) linked the type III IFNλ3 with spontaneous clearance of HCV (Ge et al., 2009; Thomas et al., 2009). Later, another study proved peripheral blood pDCs to be the main IFNλ3 producer, thus, connecting indirectly pDCs with HCV clearance (O'Connor et al., 2014; Stone et al., 2013), and suggesting an important role of pDCs in HCV clearance by another mean than IFN-I production. However, pDCs and its ability to produce large amounts of IFN and other proinflammatory cytokines may be detrimental during chronic infection because of the induction of a permanent inflammation that can ultimately lead to liver cirrhosis (Doyle et al., 2019). In conclusion, pDCs have been demonstrated to be a key player during the different phases of HCV infection, firstly involved in the clearance of HCV and secondly involved in the promotion of a proinflammatory state of the liver. Further studies are needed in order to shed light into the regulatory mechanisms of pDCs activation in HCV infection.

3. MATERIALS AND METHODS

3.1. Ethics statement

This study was conducted according to the principles expressed in the declaration of Helsinki. Each patient enrolled in TIM-3 project provided informed written consent to participation in this study in accordance with institutional and regulatory guidelines. The study was approved by the Institutional Ethics Committee (Review Board) *Na Bulovce* Hospital in Prague, Czech Republic, registration number 22.3.2013/6637/EK-Z (22 March 2013). Peripheral blood mononuclear cells (PBMCs) from healthy anonymous donors were obtained from the *Etablissement Français du Sang* (EFS). Blood samples were obtained after written consent following the approval of the EFS, Marseille, France and the *Centre de Recherche en Cancérologie de Marseille* (CRCM) in accordance to the convention signed the 20th May 2014.

3.2. Inhibitors, antibodies and reagents

MEK-1/2 inhibitor PD0325901 was obtained from *InvivoGen* (Toulouse, France) and U0126 was obtained from *Sigma* (*Sigma-Aldrich*, Lyon, France) Both were used as recommended by supplier. PD0325901 is a selective non-ATP-competitive allosteric MEK1/2 inhibitor with *in vitro* IC_{50} = 0.33 nM, which was shown to be specific against a panel of 70 different kinases at 10 µM range (54). U0126 inhibits MEK 1/2 with an *in vitro* IC_{50} of 0.5 µM. JNK inhibitor SP600125, TBK1 inhibitor BX795, NF-κB inhibitor Bay11-7082, p38 MAPK inhibitor SB253080, and calcineurin inhibitor FK506 were all purchased from *InvivoGen* (San Diego, USA). For *in vitro* pDC stimulation assays, CpG-A (ODN 2216), CpG-B (ODN 2006), and PMA were purchased to *InvivoGen* (San Diego, USA), BDCA-2 antibody was purchased to *Miltenyi Biotech* (Paris, France) and ILT-7 antibody was adquired from *eBioscience*. Syk kinase inhibitor AB8779 was from *AB Science* (Paris, France). In vitro, AB8779 was shown to be as potent as Fostamatinib (R406) with IC_{50} = 0.04 µM. For in vitro pDC stimulation assays CpG-A (ODN 2216), CpG-B (ODN 2006), resiquimod (R848), PMA (all *InvivoGen*, San Diego, USA), BDCA-2 mAb (*Miltenyi Biotech*, Paris, France), ILT-7 (CD85g) mAb and IgG1 isotype control antibody (*eBioscience*) were used.

3.3. Patients and healthy donors

Twenty-one viremic individuals with plasma viremia ≥ 10^4 RNA copies per milliliter of plasma were enrolled for a period of 9 months at the HIV Clinic of Hospital *Na Bulovce* **(Table 5)** together with 16 sex-matched controls. The difference in age distribution of HIV-1-infected individuals (median (interquartile range (IQR))) 28, IQR (25–37) years and healthy controls 34, IQR (31–37) years was not statistically significant (t-test, p = 0.26). The first day of therapy and at the same time the first day of the blood sampling was determined individually for each patient according to recommended therapeutic criteria. Blood samples (10 mL) were collected before and after suppression of viral load by antiretroviral therapy, as shown in Table 5. We had access to the clinical data of these patients including analyses of their lymphocyte populations for another 14 months. Enrolment criteria: HIV-1 infection, ≥ 10^4 HIV-1 viral copies/mL of plasma, treatment-naive state. Exclusion criteria: < 18 years, HCV coinfection (patients must be HCV PCR negative). The efficiency of ART was determined using a COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, version 2.0 (*Roche*, Basel, Switzerland).

Subject No. [1]	Transmission [2]	Age	Diagnosis-Initiation of ART (Months)	Therapy Regimen [3]	$CD4^+$ T Cells (Cell/mm^3) 0-Month ART	$CD4^+$ T Cells (Cell/mm^3) 3-Month ART	HIV-1 RNA (Copies/mL) $VL_{0\text{-month}}$ [4]	HIV-1 RNA (Copies/mL) $VL_{3\text{-month}}$ [5]
1	MSM	26	4	ABC/3TC+RPV	336	498	44,800	44
2	MSM	23	3	ABC/3TC+EFV	468	599	44,600	<20
3	MSM	25	52	ABC/3TC+DRV/r	565	676	50,600	102
4	MSM	22	10	TDF/FTC+EFV	514	624	92,100	34
5	MSM	27	10	ABC/3TC+DRV/r	527	644	153,000	34
6	Bi	44	8	ABC/3TC+EFV	480	530	82,700	52
7	MSM	26	12	TDF/FTC/RPV	1023	1111	6150	<20
8	MSM	27	5	TDF/FTC+DRV/r	315	438	144,000	850
9	MSM	38	15	TDF/FTC/EVG/c	521	598	27,300	34
10	Bi	49	62	ABC/3TC+DRV/r	379	619	119,000	67
11	Bi	48	6	ABC/3TC+DRV/r	372	501	20,200	<0
12	MSM	29	4	ABC/3TC+LPV/r	267	546	83,900	<20
13	MSM	31	1	TDF/FTC/RPV	402	528	47,300	81
14	MSM	37	13	TDF/FTC/EVG/c	634	782	99,700	61
15	MSM	36	11	TDF/FTC+DRV/r	503	597	180,000	<20
16	MSM	28	32	TDF/FTC+DRV/r	206	217	98,100	135
17	MSM	24	14	TDF/FTC/RPV	377	414	35,400	73
18	MSM	19	4	ABC/3TC+DRV/r	418	418	109,000	391
19	MSM	26	21	TDF/FTC/RPV	534	540	43,900	28
20	MSM	44	4	TDF/FTC+DTG	538	585	39,900	166
21	MSM	28	25	TDF/FTC+DTG	384	404	13,400	<20

[1] All subjects were males; [2] MSM (men who has sex with men), Bi (bisexual); [3] ABC (Abacavir); 3TC (lamivudine); TDF (tenofovir); FTC (emtricitabine); RPV ; EFV (efavirenz); DRV (darunavir); EVG (elvitegravir); LPV (lopinavir); DTG (dolutegravir); r (ritonavir) and c (cobicistat) are pharmacokinetic enhancers; [4] HIV-1 virus load (plasma HIV-1 RNA (copies/mL)) at time zero of ART; [5] HIV-1 virus load (plasma HIV-1 RNA (copies/mL)) 3 months after initiation of ART.

Table 5: Clinical characteristics of the HIV patients cohort

3.4. PBMC isolation

Patients' PBMCs were separated using a BD Vacutainer CPT™ Cell Preparation Tube (*BD Medical*, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, PBMCs were separated by density gradient centrifugation, then washed twice with PBS and used in the ensuing experiments.

3.5. Isolation and culture of primary pDCs

pDCs purified from PBMCs as described previously were 75–95% pure, with a contamination of less than 5% mDCs (Decalf et al., 2007; Dental et al., 2012; Gondois-Rey et al., 2009; Shiina & Rehermann, 2008). Isolated pDCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS). To optimize viability in overnight experiments, recombinant IL-3 (*R&D Systems Europe, Ltd.*, Abingdon, UK) was added to a final concentration of 10 ng/mL.

3.6. pDC line GEN2.2

Human pDC line GEN2.2 (Laurence Chaperot et al., 2006) was grown in a RMPI 1640 medium supplemented with L-glutamine, 10% FCS, 1% sodium pyruvate, and 1% MEM nonessential amino acids, on a monolayer of the murine stromal feeder cell line MS-5 grown in RPMI 1640 supplemented with L-glutamine, 10% FCS, and 1% sodium pyruvate. For the measurement of cytokine production, and other non-shown experiments, GEN2.2 cells were separated from the MS-5 feeder cells.

3.7. In vitro pDC stimulation

The pDC subset within PBMCs aliquoted in 100 µL quantities (10^7 cells/mL) was cultured in 96-well round-bottom culture plates and when necessary, exposed to BDCA-2 mAb before TLR stimulation. To determine cytokine production, purified primary human pDCs (in the presence of IL-3) or GEN2.2 cells were kept at a concentration of 10^6 cells/ml aliquoted in 100 µl quantities

in 96-well round-bottom culture plates and exposed to/stimulated with 4 µg/ml CpG-A or CpG-B, 25 ng/ml PMA, 20 µg/ml of BDCA-2 or ILT-7 antibody, or 10 HCV geq/cell for 16 h.

3.8. Flow cytometry analysis

To carry out the flow cytometric analysis of pDC phenotype, we designed a multicolor panel composed of PerCP/Cy5.5-CD11c, BV421-BDCA2, APC-TRAIL, PE-CD4, APC-Fire750-TIM3, FITC-Lin1 (all from *Biolegend*, San Diego, CA, USA), and V500-HLADR, BV605-CD32 (from *BD Biosciences*, San Jose, CA, USA). The staining was performed in Brilliant Stain Buffer (*BD Biosciences*) as recommended by the manufacturer. Cells were fixed in 4% paraformaldehyde and data was acquired within 48 h. We included Lin1-FITC-labeled antibody along with Zombie Green fixable viability dye (*Biolegend*, San Diego, CA, USA) in a dump channel. We used an LSR Fortessa SORP (*Becton Dickinson*, San Jose, CA, USA) cytometer equipped with 5 non-colinear lasers and 20 detectors. A final analysis of flow cytometry data was carried out using FlowJo software (*Tree Star, Inc.*, Ashland, OR, USA). Routine analyses of the major lymphocyte populations (FITC-A-CD3, PerCP-Cy5.5-CD45, PE-Cy7-CD4, APC-Cy7-CD8, APC-CD19, PE-CD16+56) in peripheral blood of ART-treated HIV-1-infected individuals were performed using a BD FACSCanto II flow cytometer (*Becton Dickinson*).

3.9. Production and purification of cell culture-derived HCVcc (JFH-1 3M) and HBV

JFH-1 3M HCVcc particles were prepared and purified as described previously (Florentin et al., 2012; Gondois-Rey et al., 2009). HBV particles were concentrated from HBV stably transfected HepG2 cell line, clone 2.2.15 (HepG2.2.15) as described previously (Luangsay et al., 2015). The 8% PEG8000 precipitated HBV supernatant purified by ultracentrifugation through 20, 30, 40, 50% sucrose was resuspended in RPMI 1640 medium to obtain a virus suspension containing 10^{12} HBV RNA copies/mL.

3.10. Determination of secreted IFN-α, TNF-α and IL-6

The quantities of total IFN-α, TNF-α, and IL-6 produced by pDCs or GEN2.2 were measured in cell-free supernatants using human ELISA kits (IFN-α and IL-6 from *Mabtech*, and TNF-α from *BD Biosciences*). The index of synergism was determined from the following formula: the level of cytokine production after stimulation with the combination of CpG and PD0325901 divided by the sum of cytokine production level after stimulation with CpG and PD0325901 separately. PD0325901 alone did not induce a detectable quantity of respective cytokines. Combinations resulting in an index of synergism > 1.5 were considered to be synergistic. The combinations resulting in an index of synergism ≤ 1.5 and in a 30% increase in stimulation compared to the stimulation observed with either of the two stimulators were considered to be additive.

3.11. Statistical analysis

Quantitative variables are expressed as the means ± SEM (standard error of the mean). To compare the levels of cytokine production by pDCs, Mann-Whitney two-tailed non-parametric test was used. Data were analyzed with GraphPad Prism 4 software (*GraphPad Software*, La Jolla, CA). p-value of 0.05 was considered to be significant.

4. RESULTS AND DISCUSSION

4.1 Expression of TIM-3 on plasmacytoid dendritic cells as a predictive biomarker of decline in HIV-1 RNA level during ART

4.1.1. Persistent dysfunction of pDCs from ART-treated HIV-1-infected individuals after sustained suppression of HIV-1 RNA

First, we analysed the main immune populations from peripheral blood of a cohort of 21 HIV-1-infected individuals **(Table 5)** and 15 healthy controls (healthy donors). To explore the dynamics of the lymphocyte populations the samples were taken and analysed every 3 months during a period of 23 months **(Figure 14)**, being the month 0 treatment naïve HIV-1-infected individuals and the starting point of the anti-retroviral therapy (ART). After 3 months of therapy the levels or HIV-1 RNA in plasma decreased dramatically from (median (interquartile range (IQR))) 4.70, (4.57-5.02) log_{10} copies/mL to 1.64, IQR (0.7-1.96) log_{10} copies/mL, and it continued decreasing during the remaining 6 months **(Figure 14)**.

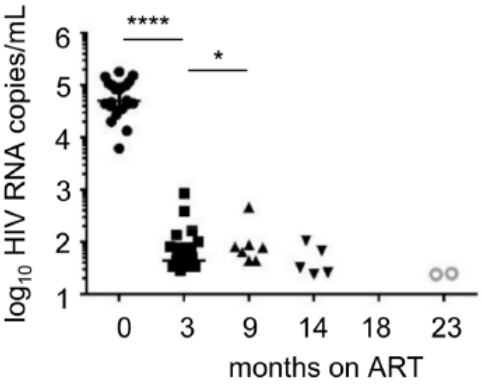

Figure 14. HIV RNA copies/mL of plasma before and after anti-retroviral therapy (ART)

Then, the main immune cell populations ($CD4^+$T lymphocytes, $CD8^+$T lymphocytes, B lymphocytes and NK cells) in treatment naïve HIV-1 infected patients, ART-treated HIV-1 infected patients, and healthy donors were quantified by flow cytometry **(Figure 15A-C)**. $CD4^+$ T cell numbers increased substantially from 469, IQR(375–531) CD4+ T cells/mm3 of blood to 748, IQR (609–945) CD4+ T cells/mm3 of blood **(Figure 15D)** at the end of the follow up (9 months

under ART). Similarly, B cell count on blood boosted from 159, IQR (137–224) B cells/mm3 of blood to 214, IQR (136–367) B cells/mm3 of blood **(Figure 15E)** as well as NK cell number that went from 230, IQR (169–459) NK cells/mm3 of blood to 412, IQR (308–541) NK cells/mm3 of blood **(Figure 15F)**. On the contrary we found that $CD8^+$T cell count diminished as the treatment went on from 1551, IQR (1070–1737) CD8 T cells/mm3 of blood to 1005, IQR (713–1555) CD8 T cells/mm3 of blood **(Figure 15G)**. 9 months after the initiation of ART the numbers of B cells in patients were still significantly higher than healthy donors'.

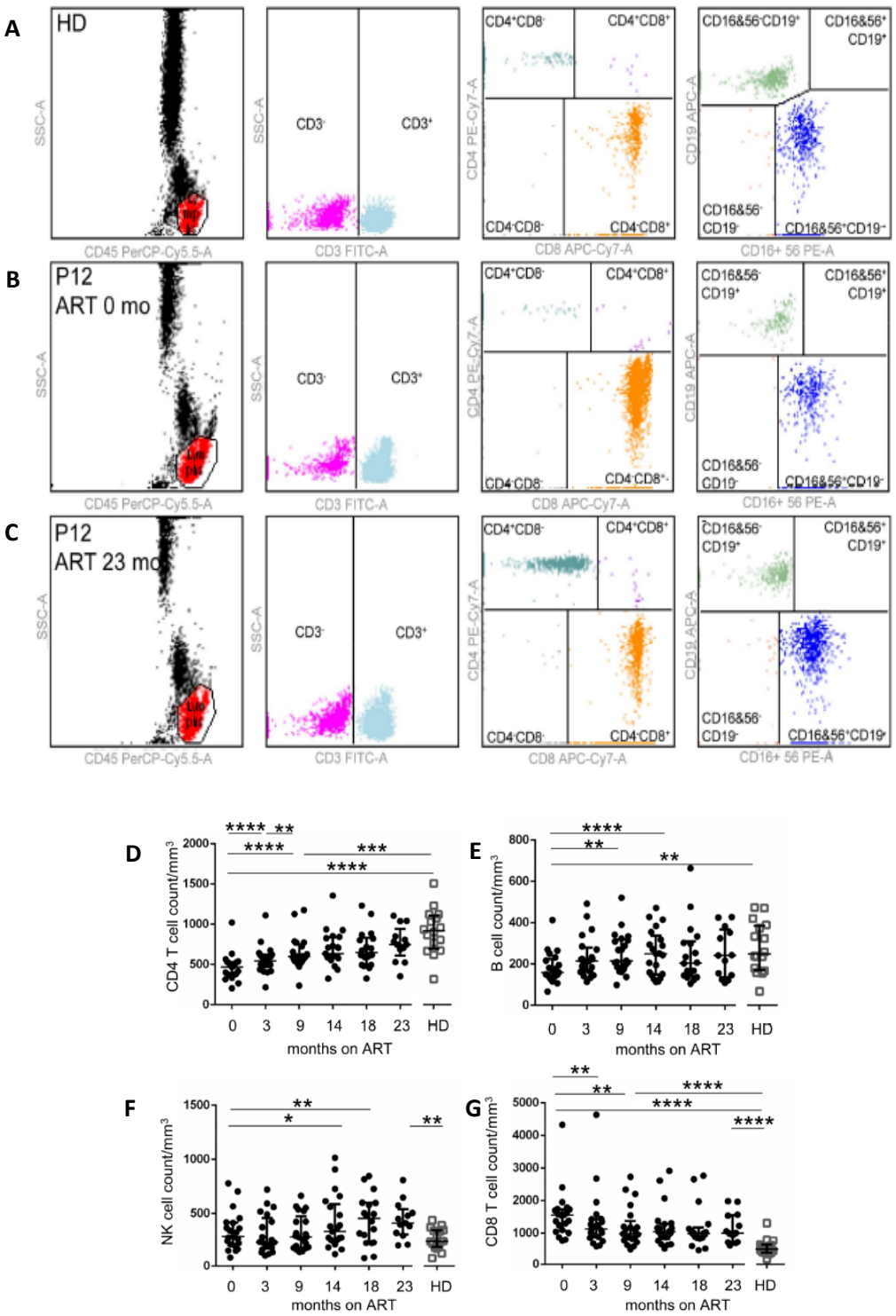

Figure 15. Adaptive immunity is only partially restored over the course of ART despite the sustained suppression of HIV-1 RNA level. Characteristics of the cohort of 21 HIV-infected subjects. *(A-C) Dot plots for the quantification of the major lymphocyte populations in peripheral blood of a healthy donor (HD) (A), treatment-naïve patient no.12 (12) (B), the same patient after 23 months of ART (C). (D) $CD3^+CD4^+$ T cell counts during ART. (E) $CD19^+$ B cell counts during ART. (F) $CD3^-CD16^+CD56^+$ NK cell counts during ART. (G) $CD3^+CD8^+$ T cell counts during ART. The data show medians and interquartile range, N=21. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; two-tailed paired Wilcoxon test.*

Contrarily to the abundance of studies referring to the impairment of the main immune populations within a HIV-1 infection, little is known about the dysfunction of pDCs. According to the design of this study, we quantified them and followed the development of the phenotype during 9 months of ART by flow cytometry. Preceding reports showed that the number of cells is markedly decreased in HIV-1 infected individuals (Kaushik et al., 2013; Soumelis et al., 2001). To assess that, we first gated the PBMC population according to their size and then excluded the doublets. After the exclusion of dead cells, pDC were defined as live $Lin^{-}CD4^{+}BDCA\text{-}2^{+}$ cells **(Figure 16A)**. As expected, we found that the number of pDCs in treatment naïve HIV-1-infected individuals (4.08, IQR (2.59–4.90) pDC/mm3) was significantly lower (54% less) than the number of pDCs in 13 healthy donors **(Figure 16B)**. Nine months after the beginning of ART the concentration of pDCs in blood was increased but its median value (5.35, IQR (3.45–7.99) pDC/mm3) remained significantly lower than that detected in healthy donors (71%, p = 0.04). There was a similar proportional increase of numbers in pDCs (1.31 times, $p = 0.04$, **Figure 16B**) as well as in $CD4^{+}$ T lymphocytes (1.28 times, $p = 0.008$, **Figure 15D**).

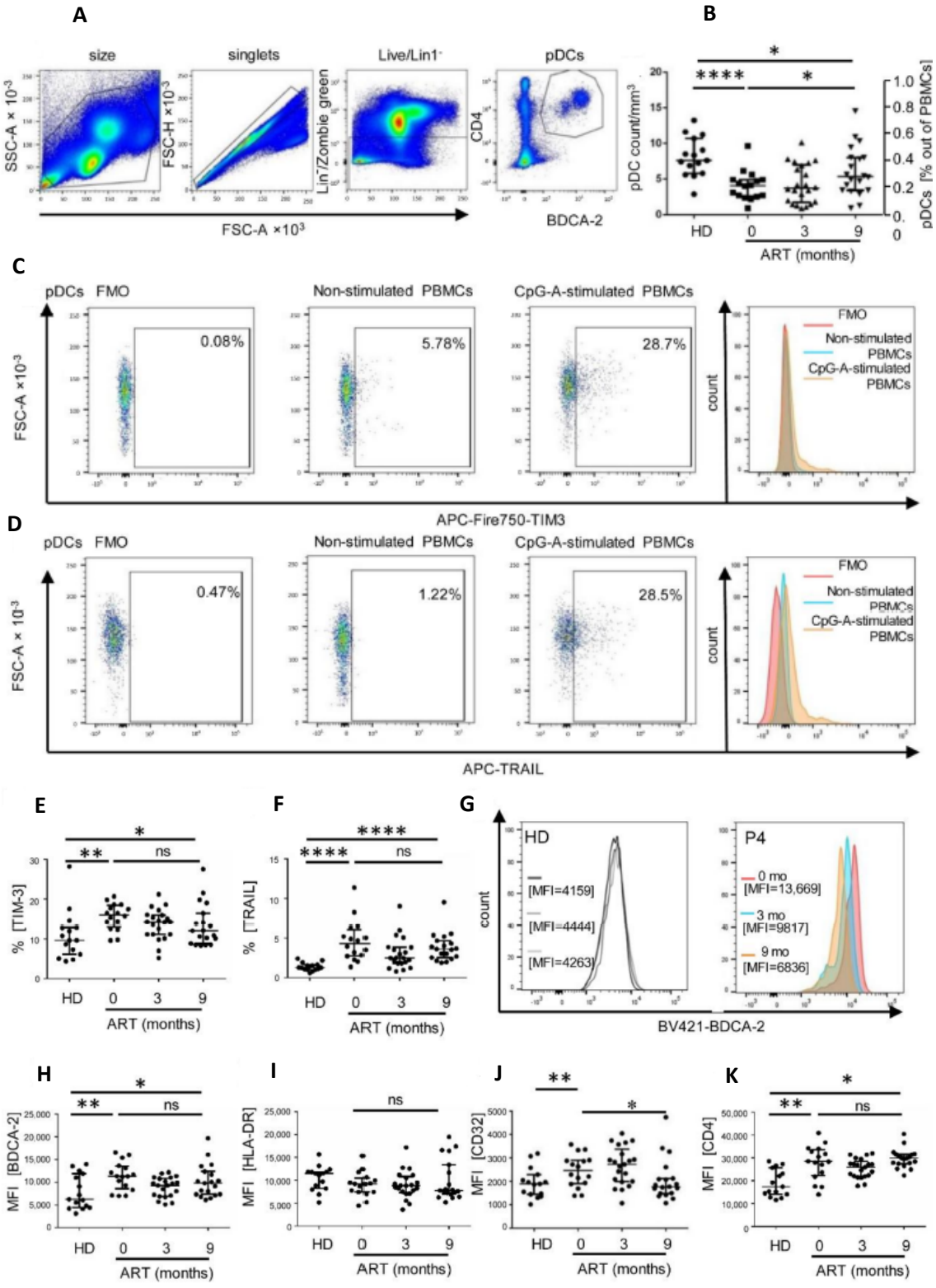

Figure 16. Impaired pDC phenotype persists after sustained suppression of HIV RNA in plasma of ART-treated patients. *(A) Gating strategy for identification of pDC phenotype: PBMCs were gated according to their size and then into singlets, and after exclusion of dead cells (Zombie green) and Lin1⁺ cells into a CD4⁺BDCA-2⁺ pDC population. (B) pDC number and proportion of PBMCs in healthy donors (HD) and in the course of ART. (C,D) Dot plots and histograms for the quantification of TIM3 (C) and TRAIL (D) in Lin⁻CD4⁺BDCA-2⁺ live pDCs are shown. FMO was used*

*for gating TIM3$^+$ (C) and TRAIL$^+$ (D) pDCs in mock-stimulated or CpG-A-stimulated PBMCs from a healthy donor and from HIV-1-infected patients (E,F). (E) The frequency of pDCs expressing TIM3 in the cohort of 21 patients. (F) The frequency of pDCs expressing TRAIL. (G) Examples of histograms for the quantification of BDCA-2 in three healthy donors (HD) and ART-treated patient no.4 (P4) determined 0, 3, and 9 months after therapy initiation. (H) The MFI of BDCA-2 expressed on pDCs. (I) The MFI of HLA-DR expressed on pDCs. (J) The MFI of CD32 expressed on pDCs. (K) The MFI of CD4 expressed on pDCs. The data show medians and interquartile range, N=21. * p < 0.05; ** p < 0.01; **** p < 0.0001; ns, non-significant; two-tailed Mann-Whitney* test.

Then we looked at the expression of phenotypic markers TIM-3, TRAIL, BDCA-2, HLA-DR, CD32, and CD4 in Lin$^-$CD4$^+$BDCA-2$^+$ pDCs **(Figure 16C–K)** in order to check the functionality of the cells. To select for positivity in TIM-3 and TRAIL we used a fluorescence minus one control (FMO) strategy. To define the positivity of the constitutive pDC markers (BDCA-2, HLA-DR, CD4) as well as CD32 we looked at the median fluorescence intensity (MFI). We found that the presence of TIM-3$^+$ pDCs in treatment-naïve individuals was 1.42 times higher than the frequency of TIM-3$^+$ pDCs in healthy donors (p = 0.0026, **Figure 16E**). After 9 months of treatment the frequency of TIM-3$^+$ pDCs decreased to 1.2 times the healthy donors' value (p = 0.0155), with a clear tendency to the restoration of the healthy values. Similarly, the frequency of TRAIL$^+$ pDCs in treatment naïve HIV-1-infected individuals was 3.32 times higher than in healthy donors (p < 0.0001), which showed as well a decreasing tendency over the 9 month follow up **(Figure 16F)**. Even though, the median values of the frequency of TIM-3$^+$ pDCs (and of the frequency of TRAIL$^+$ pDCs of the HIV-1 infected patients after 9 months of ART remained higher than the healthy donors' values. Regarding the constitutively expressed markers in pDCs as well as CD32, we compared the MFI (median fluorescence intensity) values between healthy donors, treatment naïve HIV-1 infected individuals and ART-treated HIV-1-infected individuals **(Figure 16G)**. As for the MFI of BDCA2 (1.8 times, p = 0.015) **(Figure 16H)**, CD4 (1.6 times, p = 0.0013) **(Figure 16K)**, and CD32 (1.5 times, p = 0.046) **(Figure 16J)** we found that treatment naïve HIV-1 infected individuals present a higher value than healthy donors. For HLA-DR though, MFI values were not significantly different neither between healthy donors and treatment naïve HIV-1 infected individuals nor between the late ones and ART-treated HIV-1 individuals at any time point **(Figure 16I)**. During the first 9 months of treatment BDCA2, CD4 and CD32 show a tendency to restoration to normal values of expression represented by the healthy donors' MFI values. Even though, CD4 and BDCA2 MFI remained significantly elevated in comparison to healthy donors. Contrarily, CD32 expression (1.38 times, p = 0.044) was restored after 9 months of treatment.

4.1.2. Decline in HIV-1 RNA level after initiation of ART correlates with expression of TIM-3 on pDCs

First, we defined the rate of decline of HIV-1 RNA over the first 3 months of ART as a new parameter to explore the success of ART in HIV-1-infected individuals. To this aim, we calculated the ratio of HIV-1 RNA copies/mL (virus load, VL) of plasma in treatment-naive individuals ($VL_{0\text{-}mo}$) to the level of HIV-1 RNA copies/mL of plasma in 3 months ART-treated individuals ($VL_{3\text{-}mo}$) **(Figure 17A)**.

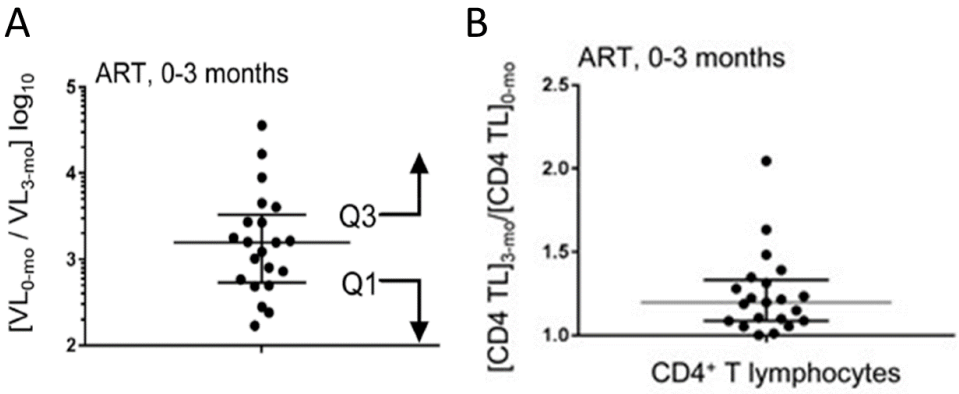

Figure 17. Rate of decline in HIV-1 virus load (VL) expressed as $[VL_{0\text{-}mo}/VL_{3\text{-}mo}]$ $\log_{10}$. *$VL_{0\text{-}mo}$ is HIV-1 RNA copy number/mL in treatment-naïve individuals (zero time of ART) and $VL_{3\text{-}mo}$ is HIV-1 RNA copy number/mL after 3 months of ART in the cohort of 21 HIV-1-infected individuals.*

We observed a marked decrease of the RNA molecules in blood 2.2-4.6 $\log_{10}$ **(Figure 17A)** while $CD4^+$ T cell number increased from 1.1 to 2.8 times **(Figure 17B)**. Importantly, the rate of decline of HIV-1 RNA copies/mL of plasma over the first 3 months of ART did not correlate with the initial viral load **(Figure 18)** nor the rate of $CD4^+$ T lymphocyte number restoration in the same individuals.

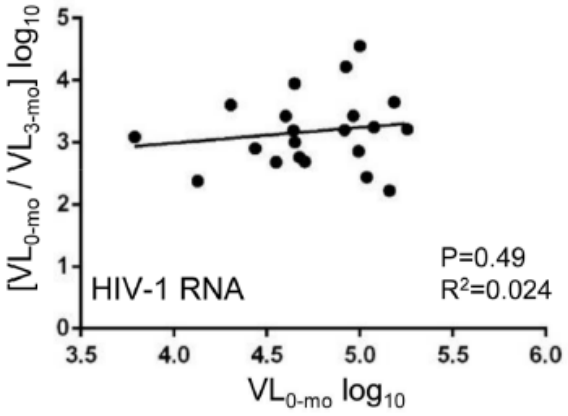

Figure 18. The rate of decline in HIV-1 RNA copies/mL does not correlate with HIV-1 VL in treatment-naïve individuals (zero time of ART).

We used the rate of decline of HIV-1 RNA copies/mL over 3 months [$VL_{0\text{-}mo}/VL_{3\text{-}mo}$] $\log_{10}$ to characterize slow and fast responding individuals to ART. After that, we assessed the expression levels of phenotypic markers in the two groups in order to explore whether a correlation can be made between these marker expression levels and the success of the ART over the first three months. Then, using the 3rd quartile (Q3) of the HIV-1 copy number decline rate as a parameter we found a significantly higher frequency of TIM-3$^+$ pDCs in the slow responders ([$VL_{0\text{-}mo}/VL_{3\text{-}mo}$] $\log_{10}$ < Q3) compared to the rapid responders ([$VL_{0\text{-}mo}/VL_{3\text{-}mo}$] $\log_{10}$ > Q3) (p = 0.015 before ART; p = 0.012 after 3-month ART) **(Figure 19 A,C)**. Then we checked the correlation between the frequency of TIM-3$^+$ pDCs with the rate of decline of HIV-1 RNA levels in plasma **(Figure 19 B,D)**. There was a significant correlation of the frequency of TIM-3$^+$ pDCs with the rate of decline of HIV-1 RNA levels in both treatment-naïve HIV-1-infected individuals **(Figure 19B)** and in HIV-1-infected individuals treated for 3 months **(Figure 19D)**. Contrarily, the correlation between the frequency of TIM-3$^+$ pDCs and the total HIV-1 RNA copy number ($VL_{0\text{-}mo}$ $\log_{10}$ copies/mL) before ART was not significant (p = 0.44).

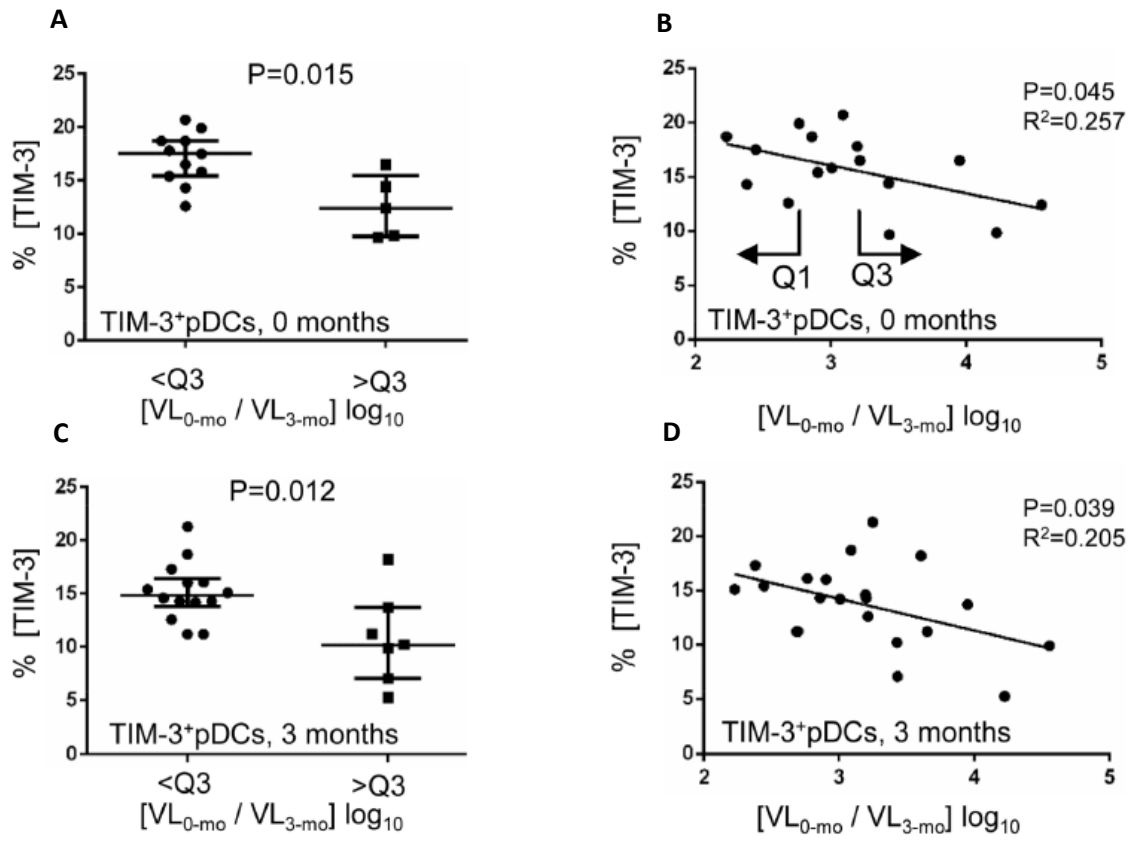

Figure 19. Expression of TIM3 on pDCs of HIV-1 infected individuals negatively correlates with the rate of decline in HIV-1 RNA copies/mL of plasma over the 3-month ART. (A,C) Comparison of the frequency of TIM3$^+$ pDCs in treatment-naïve HIV-1-infected patients (A) or patients after the 3-month ART (C), in which [$VL_{0\text{-}mo}/VL_{3\text{-}mo}$] $\log_{10}$ was <Q3 or >Q3. (B,D) Correlation of frequency of TIM3 expressed on pDCs in treatment-naïve HIV-1-infected patients (B), or patients after the 3-month ART (D) with [$VL_{0\text{-}mo}/VL_{3\text{-}mo}$] $\log_{10}$ (the same samples as in panels (A,C) were analysed). The data show medians and interquartile ranges. Q1, the first quartile; Q3, the third quartile; $p < 0.05$ was considered to be significant; two-tailed Mann-Whitney test.

Even though ART persistently suppressed HIV-1 RNA plasma levels and diminished immune activation in HIV-infected patients during the 9-month follow-up since the start of the therapy, expression levels of some of the phenotypic markers indicating immune function was not restored to the steady level observed in healthy donors (French, King, Tschampa, da Silva, & Landay, 2009; Hatano et al., 2013; Hunt et al., 2003; Ostrowski, Katzenstein, Pedersen, Gerstoft, & Ullum, 2008; Rajasuriar, Wright, & Lewin, 2015). We demonstrated the partial recovery of the adaptive immune function, as shown by the reestablishment of CD4$^+$ T cell and B cell numbers to the levels of uninfected controls. However, it didn't happen the same with the numbers of CD8$^+$ T cells and NK cells, which remained significantly over the steady level indicated by the controls (Bisset, Lung, Kaelin, Ludwig, & Dubs, 2004; Melzer et al., 2015; Shahal-Zimra et al., 2016). As reported previously, pDC numbers were significantly lower in HIV-infected patients

before the start of ART compared to healthy controls (Soumelis et al., 2001). pDC number followed a tendency of restoration to normal levels after the onset of ART and during the 9-month follow up, but the immunogenic phenotype of these cells was not restored significantly. Previous studies explored the impairment of single pDC functions (O'Brien et al., 2016; Saidi et al., 2016; J. A. Schwartz et al., 2017; Soumelis et al., 2001). In this study we performed a more complex approach by following at the same time the evolution of several markers related to the immune function on pDCs: the MHC class II ligand HLA-DR, the high affinity receptor for HIV-1 CD4, the regulatory receptor and pDC marker BDCA-2, the Fcγ receptor CD32, the marker of killer pDC function TRAIL and the pDC dysfunction marker TIM-3. Of these markers, we found that only TIM-3 correlated significatively with the rate of decline of HIV-1 RNA level after the onset of ART. We suggest that TIM-3 could be a predictive marker of the efficiency of ART given that high expression of TIM-3 on these cells indicates a relatively poor response to the treatment compared to the response when pDCs show normal levels of TIM-3 **(Figure 25)**.

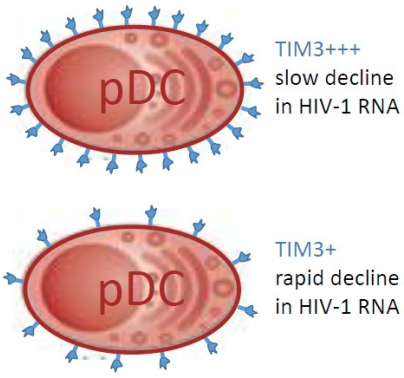

Figure 25: TIM-3 as a predictive biomarker of the efficiency of ART in HIV-1-infected patients. TIM-3 correlated significatively with the rate of decline of HIV-1 RNA level after the onset of ART.

Our study was limited by the amount of data that a small cohort of 21 HIV-infected patients could provide and the subsequent difficulty in the data analysis. Further studies with a larger cohort of patients are a must in order to explore the possibility of the usage of TIM-3 as a predictive biomarker for ART efficiency in HIV-1 infected individuals.

The mechanism by which TIM-3 is involved in the dysfunction of pDCs is still not known. Given that TIM-3 is a regulator of pDCs and its expression leads to impaired production of IFN and TNF suggests that this molecule can play an important role in the immunopathogenesis of HIV-1 infection (J. A. Schwartz et al., 2017). During HIV infection, activation of pDCs by TLR7/9 agonists

induce the expression of TIM-3 and the subsequent impairment of pDCs function. Experiments showed that TIM-3 drives the displacement of TLR9 from the submembrane region, thus, bypassing TLR activation. Interestingly, Chiba et al. suggested a mechanism in which the alarmin alarmin HMGB1, which is indispensable for TLR function, is sequestered away from the TLR preventing it from activation (Chiba et al., 2012). Moreover, TIM-3 has been shown to colocalize in $LAMP1^{+}$ acidic lysosomes with IRF7, which is an essential transcription factor involved in the production of IFN, (J. A. Schwartz et al., 2017). Apart from TIM-3, HIV-1-exposed pDCs express several dysfunction markers. Of special interest is the increased MFI of BDCA2 in pDCs from HIV-1 infected patients compared to healthy donors when it is known that BDCA2 expression is downregulated when activated in vitro by TLR7/9 agonists (Kaushik et al., 2013). From this, we can infer that non the canonical TLR activation but some other mechanism may be responsible of the increased expression of BDCA2 in HIV-1 exposed pDCs. Contrarily to the MFI, HIV-1 exposure does not influence the number of $BDCA2^{+}$ pDCs. Engagement of BDCA2 and the other pDC regulatory receptors leads to IFN-I and proinflammatory cytokines TNF and IL-6 suppression (Aouar et al., 2016; Bao & Liu, 2013; Cao et al., 2006, 2007; Dzionek et al., 2001; Hirsch et al., 2010, 2017; Swiecki & Colonna, 2015). TIM-3 may be involved also, although by different mechanisms, in the negative regulation of IFN response in order to protect the organism against the detrimental effects of it.

4.2. The MEK 1/2-ERK pathway inhibits type 1 interferon production in plasmacytoid dendritic cells

4.2.1. Mek 1/2 inhibitor potentiates CpG-A-induced production of IFN-α in pDC cell line GEN2.2

In order to restore TLR7/9-mediated-IFN-α production blocked by the engagement of RRs, we searched for inhibitors of the kinases involved in the BCR signalling that would not inhibit TLR7/9 signalling. We selected a panel of inhibitors of BCR-like, MAPK, NF-κB, and calcium signalling pathways and tested their ability to modify the production of IFN-α after exposure to the TLR9 agonist CpG-A in the pDC cell line GEN2.2 (**Figures 20 A,B;** Figure S1 in Supplementary Material). Because of the difficulty of obtaining and maintain in culture the short-living human primary pDCs, we decided to use the human pDC cell line GEN2.2, which has a shared key phenotype with the primary cells.

The inhibitors of JNK(SP600125), TBK1(BX795), NF-κB(Bay11-7082), p38 MAPK(SB253080) and calcineurin (FK506) inhibited strongly the production of IFN-α. Contrarily, the inhibitor of MEK1/2 (PD032590) potentiated the production of IFN-α in comparison with the control sample (p = 0.0022, **Figure 20B**). Several independent experiments (N = 34) were performed showing that the production of IFN-α in CpG-A-stimulated GEN2.2 cells was increased 2.55 ± 0.63 times (mean ± SEM, $p < 0.0001$), from 18.4 ± 1.4 ng/mL in the absence of the inhibitor to 44.2 ± 2.7 ng/mL when the inhibitor PD032590 was present at 1 μM (Figure S2 in supplementary material). In spite of the high variability of IFN-α production by GEN2.2 when challenged only with CpG-A, our tests were highly reproducible respect to the proportional increase of the production of IFN-α when PD032590 was also present. Apart from IFN-α, IL-6 production was increased as well when MEK1/2 was present **(Figures 20 C,D)** but this was not the case of TNF-α production, which was totally inhibited at a concentration of 0.1 μM of PD032590 **(Figure 20 E)**. This result suggests a direct involvement of MEK1/2-ERK in the TNF-α pathway. At a MEK1/2 concentration ≥ 0.01 μM and a CpG-A concentration of 4 μg/ml we observed the strongest synergistic effects (synergistic index >3) on the production of IFN-α. Regarding IL-6 production, the synergistic index obtained was >2. In contrast to what we observed when using a concentration of MEK1/2 inhibitor ≥ 0.01, the combination of 0.001 μM MEK1/2 inhibitor and 4 μg/ml CpG-A had only an additive effect on the production of IL-6 **(Figure 20 D)**. In the control experiment using PMA, TNF-α was produced but not IFN-α nor IL-6. TNF-α secretion was strongly supressed by MEK1/2

inhibitor **(Figures 1F–H)**. This results indicate that the CpG-A-induced TLR-9-mediated production of IFN-α and IL-6 are potentiated by MEK1/2 inhibitor PD0325901.

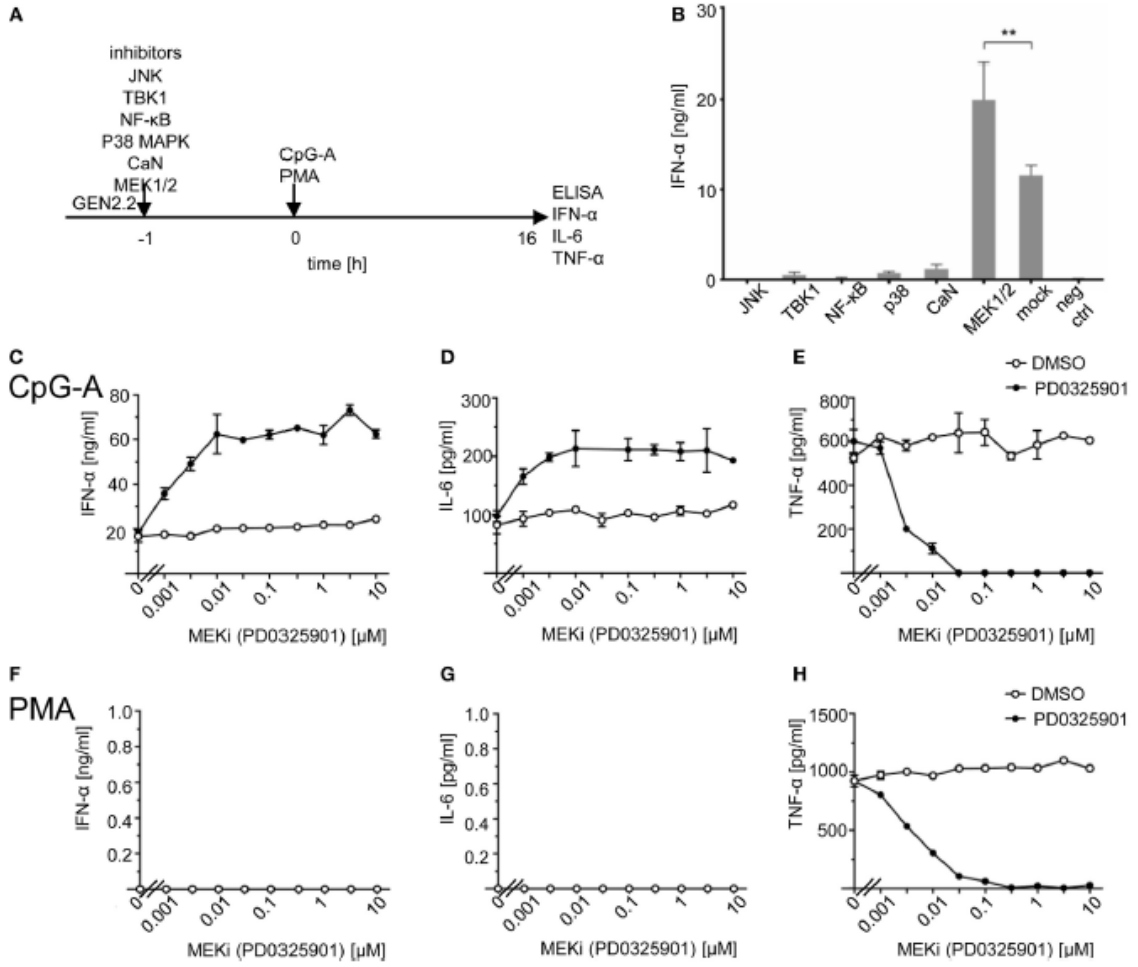

Figure 20. Effect of MEK1/2 inhibitor PD0325901 on cytokine production in CpG-A and phorbol myristate acetate (PMA)-stimulated GEN2.2 cells. *(A) Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were exposed or not to inhibitors of Jun N-terminal kinase (JNK), TANK binding kinase 1 (TBK1), NF-κB, p38 MAPK, calcineurin or MEK1/2 for 1h and then stimulated with CpG-A at 4 ug/ml. The concentrations of IFN-α, IL-6 and tumor necrosis factor α (TNF- α) in the cell-free supernatant was determined by ELISA after a 16h treatment. (B) The production of IFN- α by GEN2.2 cells stimulated with CpG-A in the presence of JNK (SP600125, 10 μM), TBK1 (BX795, 1 μM), NF-κB (Bay11-7082, 1 μM), p38 MAPK (SB253080, 1 μM), calcineurin (FK506, 0.1 μM) or MEK1/2 (PD0325901, 1 μM) inhibitors. The PD0325901 concentration-dependent production of IFN-α (C,F), IL-6 (D,G), and TNF-α (E,H) in CpG-A-induced (C-E) or PMA-induced (F-H) GEN2.2 cells. The data show mean and SEM of two independent experiments in biological triplicates (B-H). ** p < 0.01; two-tailed Mann-Whitney test.*

4.2.2. Mek 1/2 inhibitors partially restore TLR9-mediated IFN-α production blocked by ligation of RRs with BDCA2 and ILT-7 mAbs

Here, we investigated the capacity of PD0325901 to reverse the inhibitory effect produced by the ligation of RR in the production of TLR9-mediated IFN-α in GEN2.2 and primary pDCs. We first treated GEN2.2 and primary pDCs with MEK1/2 inhibitor and subsequently exposed them to 5 µg/ml of BDCA-2 mAb before challenging the cells with TLR9 agonist CpG-A **(Figure 21A)**. In GEN2.2, the production of IFN-α was supressed to 13% (p = 0.0006, **Figure 21B**) by BDCA-2 mAb in the cells that were exposed to CpG-A but not treated with PD0325901. As previously shown **(Figure 20C)**, production of IFN-α was significantly increased (3.8-fold, N = 6, p = 0.0022, **Figures 21B,C**) by PD0325901 in GEN2.2 after activation by CpG-A. Moreover, PD0325901 released the inhibitory effect provoked by BDCA-2 mAb in the production of IFN-α in GEN2.2, rising the IFN-α level up to the one found in control treatment without the MEK1/2 inhibitor (7.3-fold, p = 0.0022, **Figure 21C**). As observed in GEN2.2 cells, the exposure of primary pDCs from healthy donors to BDCA-2 mAb suppressed the production of IFN-α induced by CpG-A to 11.5% (N = 9, p = 0.0039, **Figure 21D**). Contrarily to GEN2.2, no potentiation was observed in TLR9-mediated production of IFN-α by primary pDCs when they were previously treated with PD0325901 in the absence of BDCA-2 mAb **(Figures 21B-E)**. Even though, IFN-α production was increased in a similar way to GEN2.2 when primary cells were pre-treated with BDCA-2 mAb apart from the MEK1/2 inhibitor **(Figures 21D,E)**. PD0325901 significantly restored the production of IFN-α inhibited by BDCA-2 mAb (2.4-fold, p = 0.0039, **Figure 21E**). We found a similar restoration effect when PD0325901 was added to a final 10 nM concentration (Figure S3 in supplementary material). Moreover, the results obtained when using the MEK1/2 inhibitor U0126 and ILT-7 as a RRs pathway inhibitor are analogue to the previously commented results (Figure S4 in Supplementary Material). In conclusion, our results show that MEK1/2 inhibitor PD0325901 significantly increase the TLR9-mediated IFN-α production after the blockade made by RRs agonists.

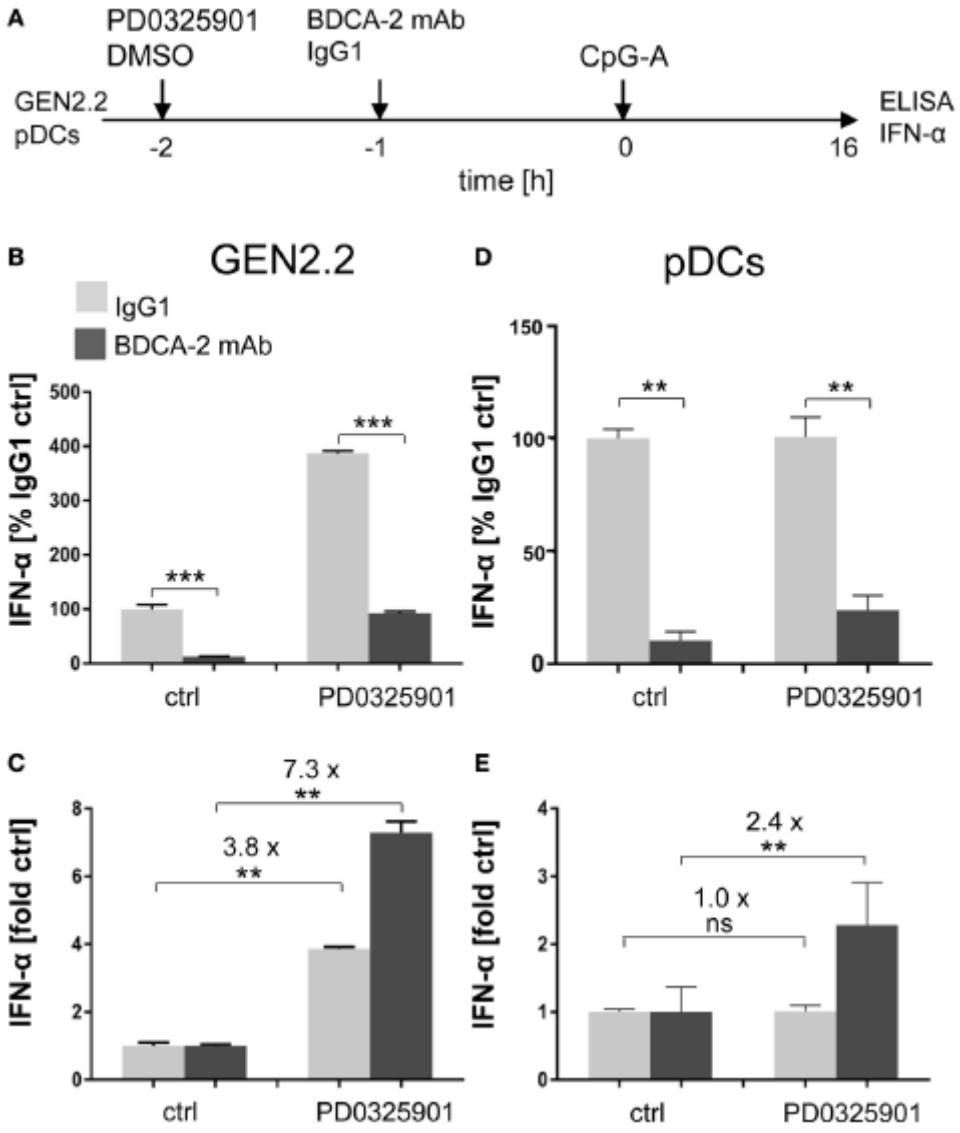

Figure 21. Effect of MEK1/2 inhibitor PD0325901 on the blockade of IFN-α production by ligation of regulatory receptors of GEN2.2 cells or primary pDCs with BDCA-2 mAb. (A) Experimental outline. GEN2.2 cells separated from MS-5 feeder cells or primary pDCs were incutabed with the MEK1/2 inhibitor for 1h before stimulation with BDCA-2 mAb and CpG-A. After a 16h culture, the IFN-α production was determined in the cell-free supernatants by ELISA. (B,D) The IFN-α production was normalized to the level induced by CpG-A in the presence of IgG1 and in the absence of the MEK1/2 inhibitor. (C,E) The same data showing the IFN-α production in panels (B-D) were normalized to the level induced by CpG-A in the absence of the MEK1/2 inhibitor. The data show mean and SEM of (B,C) six independent experiments with GEN2.2, ** $p < 0.01$; *** $p < 0.001$; two-tailed Mann-Whitney test, and (D,E) nine independent experiments with primary pDCs from different healthy donors, ** $p < 0.01$; two-tailed paired Wilcoxon test.

4.2.3. Mek 1/2 inhibitors restores TLR7/9-mediated IFN-α production blocked by HCV virions

Our group and others previously reported that some viruses, such as HCV, HBV and HIV interact with the RR BDCA-2 expressed in pDCs via the envelope glycoprotein and activates the BCR-like pathway that leads to the inhibition of the production of IFN-α. Here, we tested whether after addition of CpG-A,, the blockade of TLR9-mediated IFN-α production exerted by HCV (10 HCV geq/cell) could be released by the previous exposition to PD0325901 in the pDC cell line GEN2.2 **(Figures 22A,B)** and in primary pDCs **(Figures 22A,C)**. As expected, in the absence of PD0325901 HCV virions interfered the production of IFN-α in both GEN2.2 (35%, **Figure 22B**) and primary pDCs (34%, **Figure 22C**). We found that after exposure to PD0325901, the inhibition of TLR9-mediated IFN-α production exerted by the ligation of HCV was released and the production of IFN-α significantly increased in both GEN2.2 cells (4.2-fold, p = 0.025, **Figure 22B**) and in primary pDCs (3.2-fold, p = 0.0059, **Figure 22C**) compared to the samples where MEK1/2 inhibitor was absent. In conclusion, pharmacological targeting of MEK1/2-ERK abrogates the HCV suppression of IFN-α production.

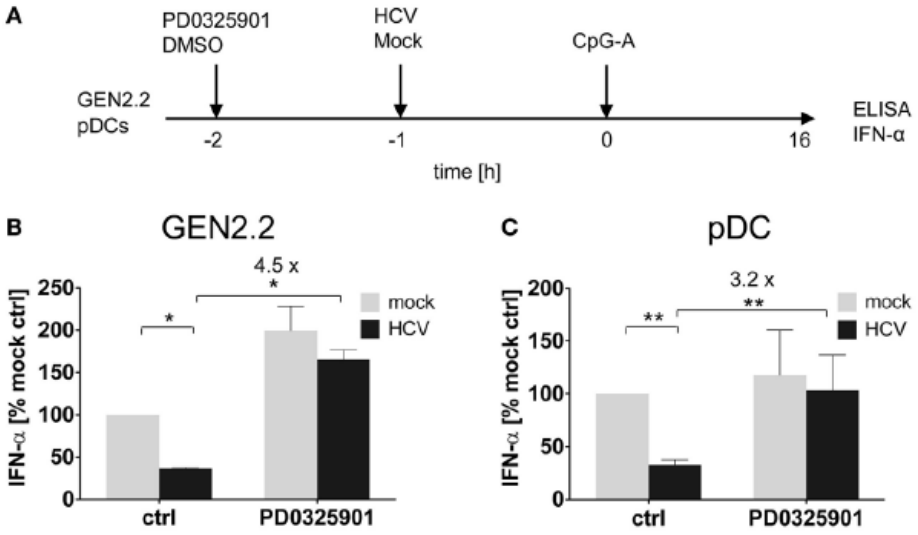

Figure 22. Effect of MEK1/2 inhibition on the hepatitis C virus (HCV) blockade of IFN-α production in GEN2.2 cells or primary pDCs. (A) Experiment outline. GEN2.2 cells separated from MS-5 feeder cells (B), or primary pDCs (C), were incubated with 1 µM MEK1/2 inhibitor PD0325901 for 1h and then treated with HCV virions at MOI = 10 geq/cell for 1h before CpG-A stimulation. After a 16h culture, the IFN-α production was determined in the cell-free supernatants by ELISA. (B,C) The IFN-α production was normalized to the level induced by CpG-A in the presence of a mock-infected control and in the absence of PK0325901. The data show mean and SEM of (B) two independent experiments with GEN2.2 cells, * $p < 0.05$; unpaired, two-

tailed t-test and (C) ten independent experiments with primary pDCs from different healthy donors, ** $p < 0.01$; two-tailed paired Wilcoxon test.

Despite intense research by several laboratories, the molecular mechanisms of the regulation of TLR7/9-mediated IFN-I and proinflammatory cytokines production in pDCs are still elusive (Bao & Liu, 2013; Cao et al., 2009, 2007; Dzionek et al., 2001; Hirsch et al., 2010, 2017; Pellerin et al., 2015; Swiecki & Colonna, 2015). We tested the effect of a panel of kinase inhibitors involved in BCR-like, MAPK, NF-κB, and calcium signalling in the production of TLR9-mediated type I IFN and proinflammatory cytokines production. While inhibitors of JNK, TBK1, NF-κB, p38 MAPK, and calcineurin abrogated drastically IFN-α production, MEK inhibitor PD032590 significantly potentiated it in GEN2.2 cells but not in primary pDCs. These results indicate that there is an intrinsic blockade of TLR9-mediated type I IFN production and that MEK is a crucial player in this interfering pathway in GEN2.2. TLR9-mediated IL-6 secretion was also potentiated by PD032590 in GEN2.2, contrarily to TNF-α, which was inhibited. These results suggest that the production of IFN-α and IL-6 is mediated by a different but related signalling pathway than the production of TNF-α. The production of TNF-α involved the participation of PKC, contrarily to the production of IFN-α and IL-6. Studies shown that ligation of RRs by different means abolished TLR9-mediated type I IFN production by means of an interfering BCR-like pathway (Aouar et al., 2016; Bao & Liu, 2013; Cao et al., 2006, 2007; Dzionek et al., 2001; Florentin et al., 2012; Gilliet et al., 2008; Hirsch et al., 2010, 2017; Martinelli et al., 2007; Swiecki & Colonna, 2015; Woltman et al., 2011; Y. Xu et al., 2009). BDCA2 mAb was the most potent inhibitor of type I IFN of a series of inhibitors (data not shown). We demonstrated that the blockade of TLR9-mediated IFN-I production by the RR BDCA2 ligation in GEN2.2 and primary pDCs is partially released when cells are pre-exposed to the MEK inhibitor PD0325901. We obtained the same results when RR are ligated by HCV virions. These results imply that MEK is a central player in the RRs pathway that regulate IFN-1 production in pDCs and that pharmacological targeting of MEK is a way to restore and potentiate IFN-I production abolished by HCV engagement. It is important to note that targeting MEK with inhibitors could be a mechanism of restoring IFN-I production blocked not only by HCV, but also by other viruses which hijack this mechanism in order to induce tolerance (Florentin et al., 2012; Hirsch et al., 2010; Martinelli et al., 2007; Woltman et al., 2011; Y. Xu et al., 2009). Further experiments are necessary to explore this hypothesis. Our results demonstrate the importance of MEK1/2-ERK signalling in the RR-mediated inhibition of IFN-α and IL-6.

4.3. Dual role of the tyrosine kinase syk in regulation of toll-like receptor signalling in plasmacytoid dendritic cells

4.3.1. Syk inhibitor blocks TLR7/9-mediated production of IFN-α and proinflammatory cytokines

We examined the effect of the Syk inhibitor AB8779 on the production of cytokines triggered by TLR9 agonsists CpG-A and CpG-B and the PKC agonist phorbol 12-myristate 13-acetate (PMA) in GEN2.2 cells **(Figure 23A)**. We found that the production of IFN-α is inhibited by AB8779 with an $IC_{50,CpG-A}$ = 0.117 μM and an $IC_{50,CpG-B}$ = 0.215 μM, TNF-α with an $IC_{50,CpG-A}$ = 0.006 μM and an $IC_{50,CpG-B}$ = 0.058 μM and IL-6 with $IC_{50,CpG-A}$ = 0.023 μM $IC_{50,CpG-B}$ = 0.021 μM **(Figure 23B)**. PMA-activated GEN2.2 cells did not produce neither IFN-α nor IL-6 but only TNF-α, which was weakly inhibited by AB8779. Moreover, we investigated the effect of AB8779 in the production of cytokines on primary pDCs from healthy donors. GEN2.2 cells do not produce IFN-α in response to synthetic agonists of TLR7, so we used primary pDCs to assess the production of cytokines triggered by both TLR9 and TLR7 agonists. AB8779 inhibited the production of IFN-α, TNF-α and IL-6 in primary pDCs when stimulated with CpG-A, as it did in GEN2.2. The abrogation of the production of these three cytokines was also observed when primary pDCs were stimulated with the synthetic agonist of TLR7 Resiquimod (R848) (Data not shown). We concluded that the Syk inhibitor AB8779 specifically blocked the TLR7/9-mediated production of IFN-α and of proinflammatory cytokines TNF-α and IL-6.

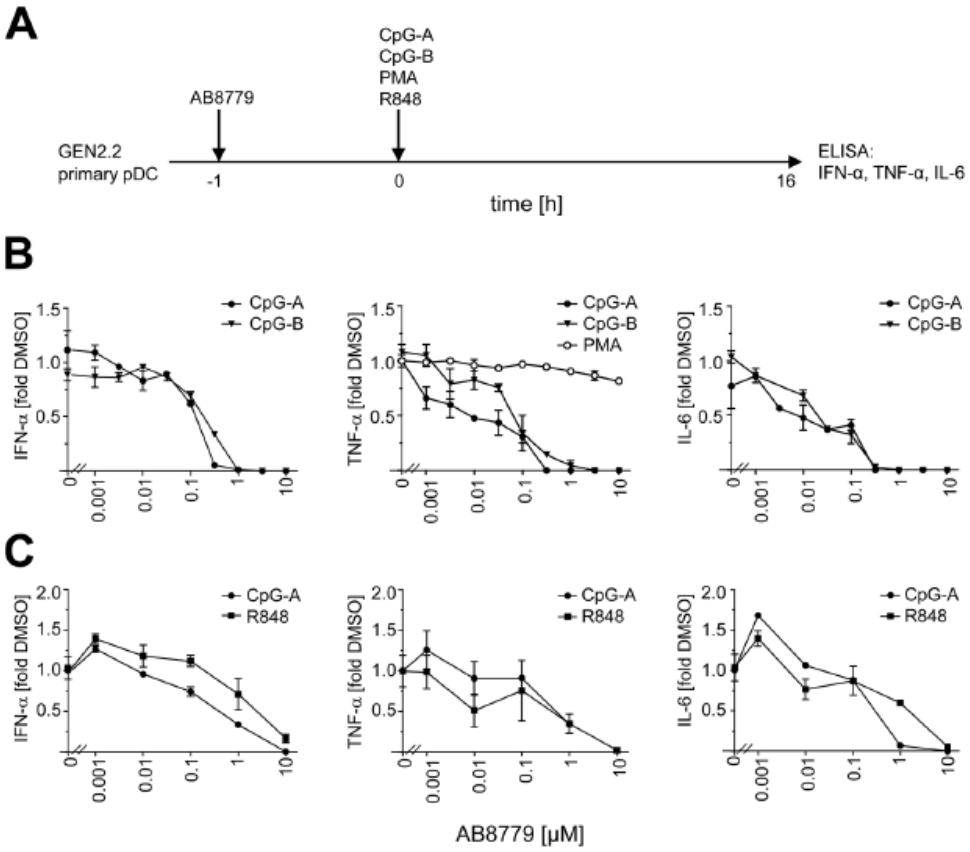

Figure 23. Effect of Syk inhibitor AB8779 on production of IFN-α and IL-6 in pDCs. (A) Experimental outline. GEN2.2 cells (B), or primary pDCs (C) were incubated with different concentrations of Syk inhibitor AB8778 for 1 hr before stimulation with CpG-A, CpG-B and PMA (N=3) (B), or CpG-A and R848 (N=2) (C). After 16 hr culture, IFN-α, TNF-α and IL-6 production in GEN2.2 cells (B) or primary pDCs (C) was determined in cell-free supernatants by ELISA and the results are expressed as a multiple of control with the matching concentration of DMSO.

4.3.2. Subliminal concentrations of Syk inhibitor enhance IFN-α production abrogated by crosslinking of RRs mAbs or virus particles

As there is an stronger inhibitory effect of AB8779 on Syk phosphorylation when induced by BDCA-2 compared to when induced by CpG-A (data not shown) , it is reasonable to think that minor concentrations of the Syk inhibitor that would block only the BCR-like pathway triggered by RR could diminish the blockade that RRs pathway induces over IFN-α production. To test whether this is true, we exposed GEN2.2 cells that were pre-treated with a relatively low concentration of AB8779 (0.01 μM) to BDCA-2 and ILT-7 agonists as well as to HCV and HBV particles before the inoculation of CpG-A to the media **(Figure 24A)**. As expected, production of IFN-α by GEN2.2 when Syk inhibitor was absent was abrogated by the RRs agonists and the viral particles **(Figure 24B)**, but when the cells were pre-treated with 0.01 μM of AB8779 the

production of IFN-α increased significantly **(Figure 24C)**. IFN-α secretion to the media increased in cells treated with ILT-7 (1.8-fold, $p = 0.03$), and in cells treated with BDCA-2 (1.6-fold, $p = 0.04$), and showed a trend to enhanced production when the cells were exposed to HCV (1.8-fold at both MOI = 1 and MOI = 3) or with HBV (1.8-fold at MOI = 0.5 and 2.3-fold at MOI = 1.3).

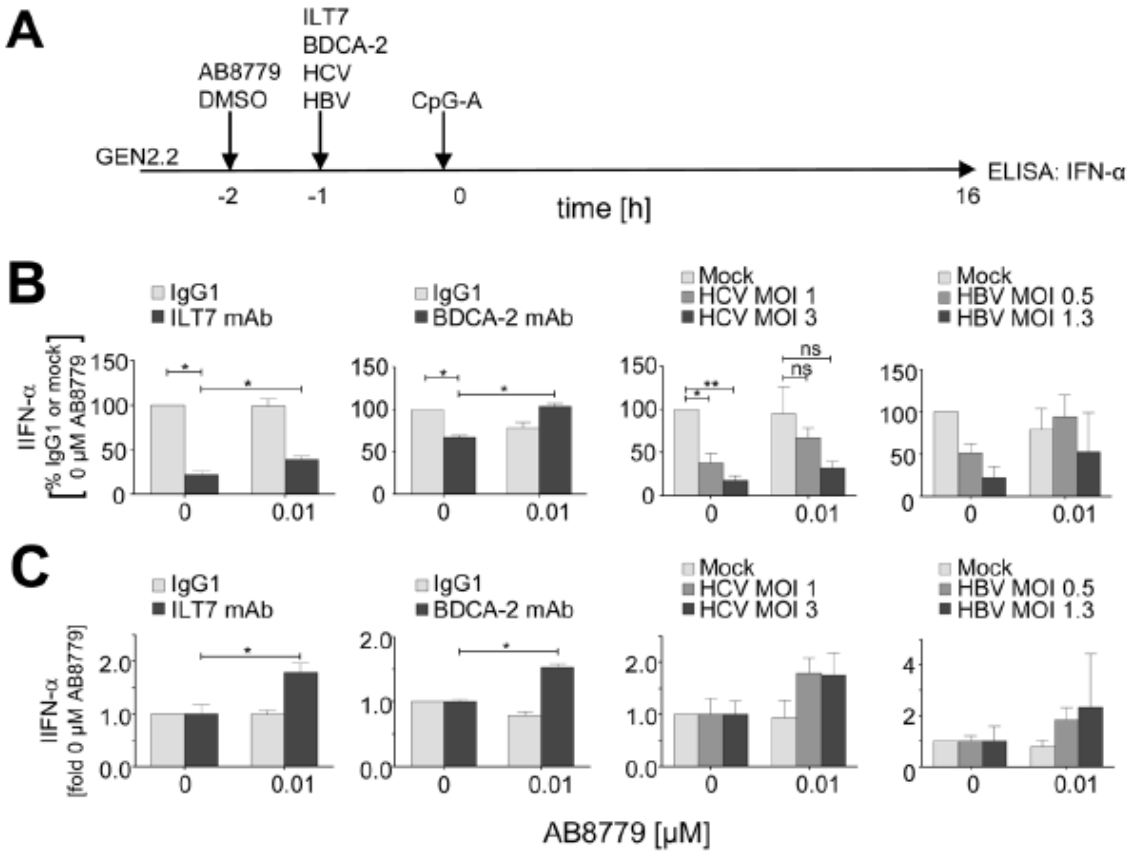

Figure 24. Subliminal concentrations of Syk inhibitor partially restore IFN-α production in GEN2.2 pDC cell line. (A) Experimental outline. After separation from MS-5 feeder cells, GEN2.2 cells were incubated with 0.01 µM AB8779 or with a matching concentration of DMSO for 1 hr before exposure to ILT-7 or BDCA-2 mAb or HCV or HBV particles and stimulation with CpG-A. (B) After 16 hr culture, IFN-α production was determined in GEN2.2 cell-free supernatants by ELISA, and the results were standardized to the quantity of IFN-α produced by GEN2.2 exposed to isotype control Ab or mock-infected culture in the absence of AB8779 (N=3). (C) IFN-α production determined in GEN2.2 exposed to ILT-7 or BDCA-2 mAb or HCV or HBV particles (shown in B) was normalized to IFN-α production in the absence of AB8779. * $p<0.05$; ** $p<0.01$.

Syk has been shown to play an important role in the IFN-I response in macrophages/monocytes since Syk function is decisive for CD14-dependent endocytosis of TLR4 and subsequent IFN-I and proinflammatory cytokines production (Lin, Huang, Chu, Lin, & Lin,

2013; Sanjuan et al., 2006; Zanoni et al., 2011). Syk has also been shown to be essential in CpG-mediated activation and differentiation of B cells (Kremlitzka, Macsik-Valent, & Erdei, 2015). In contrast, the role of Syk in pDC TLR7/9 signalling is still poorly understood. Whereas TLR9/7 signalling represents pDC immunogenic function, RR BCR-like signalling is responsible for the immunotolerant activity of these cells. We demonstrate that Syk is involved not only in the regulatory BDCA-2 signalling pathway but also in TLR 7/9-mediated signalling and in RRs pathway. Other results, show that both CpG-mediated activation of pDCs via TLR9 and anti-BDCA2 mAb-mediated activation of the RR BDCA2 induce the phosphorylation of Syk, which implies its activation (supplementary figure S9). We found that while standard concentrations (1 µM) of the Syk inhibitor AB8779 inhibit TLR7/9-mediated IFN-I production, subliminal concentrations (≤ 0.01 µM) of AB8779 increase IFN-I secretion, previously blocked by crosslinking of RRs with specific mAbs or by viral particles. These results are in consonance with the differences in phosphorylation dynamics of Syk depending on which of the two signalling pathways, TLR9 or RRs, is activated (Aouar et al., 2016). We interpret that subliminal concentrations (≤ 0.01 µM) of Syk inhibitor are able to block RRs signalling pathway in which Syk is phosphorylated before (peak at 2 min after activation), and with significantly more intensity, as shown in (Aouar et al., 2016), after RRs crosslinking compared to TLR9 pathway (peak at 30 min after activation). If Syk inhibitor concentrations increase to the standard range (1 µM) both pathways are inhibited with the subsequent inhibition of IFN-I production. Our results suggest the presence of two differentiated pools of Syk within pDCs, one controlling TLR7/9 signalling and the other controlling RRs pathway. We suggest that pharmacological targeting of Syk with standard concentrations could alleviate the symptoms caused by diseases in which there is an overproduction of IFN-I, such as systemic lupus erythematosus or psoriasis. On the other hand, subliminal concentrations of AB8779 would serve to stimulate IFN-I production in the context of a viral infection, such as HIV, HCV or HBV, preventing viral escape. We were limited by the availability of primary pDCs as well as the impossibility to work *in vivo* in our facilities. Further research on IFN-I regulatory pathways in pDCs *in vitro* and *in vivo* is a must.

5. CONCLUSIONS

5.1. Expression of TIM-3 on plasmacytoid dendritic cells as a predictive biomarker of decline in HIV-1 RNA level during ART

We performed a 9-month follow-up of 21 HIV-infected patients in order to explore the dynamics of the pDCs function by measuring expression of several phenotypic markers of immunogenic activity. We conclude that:

- The immunogenic phenotype of pDCs is only partially restored after successful suppression of HIV RNA in plasma in ART-treated HIV-infected individuals during the first 9 months of therapy.
- The levels of TIM-3 expressed in pDCs correlate negatively with the rate of decline of HIV RNA level in plasma after the initiation of ART

5.2. The MEK1/2-ERK pathway inhibits type I IFN production in plasmacytoid dendritic cells

We did a series of biochemical assays focused on the pharmacological targeting of IFN regulatory pathways in primary pDCs and in the pDC cell line GEN2.2 in order to understand the mechanisms that mediate the control of IFN production on these cells. We conclude that:

- MEK1/2 are key kinases in the regulation of IFN-α production in the pDC cell line GEN2.2 because of its participation in a constitutive/intrinsic inhibitory BCR-like pathway over TLR9-mediated IFN-α production.
- MEK1/2 inhibitor potentiates TLR9-mediated IFN-α production by abrogating the constitutive/intrinsic inhibitory BCR-like pathway over TLR9-mediated IFN-α production.
- MEK 1/2 inhibitor releases the abrogation of TLR9-mediated IFN-α production caused by the crosslinking of RRs BDCA2 and ILT-7 with mAbs or with HCV virions.

5.3. Dual role of the tyrosine kinase Syk in the regulation of toll-like receptor signalling in plasmacytoid dendritic cells

We did a series of biochemical assays focused on the pharmacological targeting of IFN regulatory pathways in the pDC cell line GEN2.2 in order to understand the mechanisms that mediate the control of IFN production on these cells. We conclude that:

- There are two differentiated pools of Syk involved in the regulation of TLR9-mediated regulation of IFN-α production. On one hand a pool of Syk is directly involved in TLR9-mediated IFN-α production. On the other hand, a pool of Syk participates in RRs signalling pathway which restricts the overproduction of IFN-α.
- Due to the different phosphorylation dynamics of the Syk pools, subliminal concentrations of the Syk inhibitor AB8779 (≤ 0.01 μM) act only on RRs Syk pool, releasing the abrogation of IFN-α production promoted by the crosslinking of RRs. Standard concentrations of AB8779 (1 μM) inhibit both Syk pools, thus, blocking TLR9-mediated IFN-α production.

6. SIGNIFICANCE OF THE RESULTS

We studied the expression of TIM-3 in parallel with other molecules characteristic of an immunogenic phenotype in pDCs in the context of an ongoing HIV infection in treatment-naïve patients and during the first 9 months of antiretroviral treatment. We discovered that TIM-3 levels on pDCs surface correlate with the efficiency of the treatment on clearing HIV-1 RNA from plasma on the first months of ART. Exploring the possibilities of TIM-3 as a putative biomarker for treatment efficiency would be interesting in order to take this information into account during the preparation of patient-specific HAART cocktails. There is still little information about the molecular mechanisms that drive the expression of TIM-3 in pDCs and further experiments would add complementary information to the issue.

We also investigated the role of the kinases Mek and Syk on the signalling pathways that lead to the production of IFN-I and its regulation. We found that independent pools of Syk are involved in the positive and negative regulation of TLR9-mediated production of IFN-I. Subliminal concentrations of Syk inhibitor targeted the RRs-mediated negative signalling pathway with the result of an augmentation of IFN-I production. On the contrary, standard concentrations of Syk inhibitors blocked both pools of Syk and the subsequent production of IFN-I. In the case of Mek, we found that it is a central kinase involved in the RRs pathway, inhibitory of IFN-I production. Blocking Mek function with inhibitors liberated the blockade over IFN-I secretion. The development of commercial pharmacological inhibitors of Syk and Mek would serve to alleviate the symptoms of autoimmune diseases such as systemic lupus erythematosus or psoriasis, as well as to modulate positively the production of IFN-I in the context of stealth chronic viral infections such as HCV, HBV or HIV.

7. INVOLVEMENT OF THE STUDENT IN THE PUBLICATIONS

The articles are shown in order of importance regarding the amount and quality of my involvement on the works.

Publication 1: ***Expression of TIM-3 on plasmacytoid dendritic cells as a predictive biomarker of decline in HIV-1 RNA level during ART.***
Shared first authorship.

- I carried out all the experimental work concerning this article, including HIV-infected and non-infected control blood collection, PBMC preparation, cell manipulation in BSL3 laboratory, antibody panel design for Flow cytometry experiments, Flow cytometry experiments, and data analysis. All my work regarding this publication is exposed in this dissertation.

Publication 2: ***The MEK1/2-ERK pathway inhibits type I IFN production in plasmacytoid dendritic cells.***
Shared first authorship.

- The experimental work in this article was performed by me and others. I performed replicates of all the variants of the *in vitro* cell stimulation and subsequent measurement of cytokine production and data analysis exposed in this dissertation. The article contains other sections in which I did not participate and are not exposed in this dissertation.

Publication 3: ***Dual role of the tyrosine kinase SYK in regulation of toll-like receptor signalling in plasmacytoid dendritic cells.***
Coauthor.

- The experimental work in this article was performed by me and others. I performed replicates of all the variants of the *in vitro* cell stimulation and subsequent measurement of cytokine production and data analysis exposed in this dissertation. The article contains other sections in which I did not participate and are not exposed in this dissertation.

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9. SUPPLEMENTARY MATERIALS

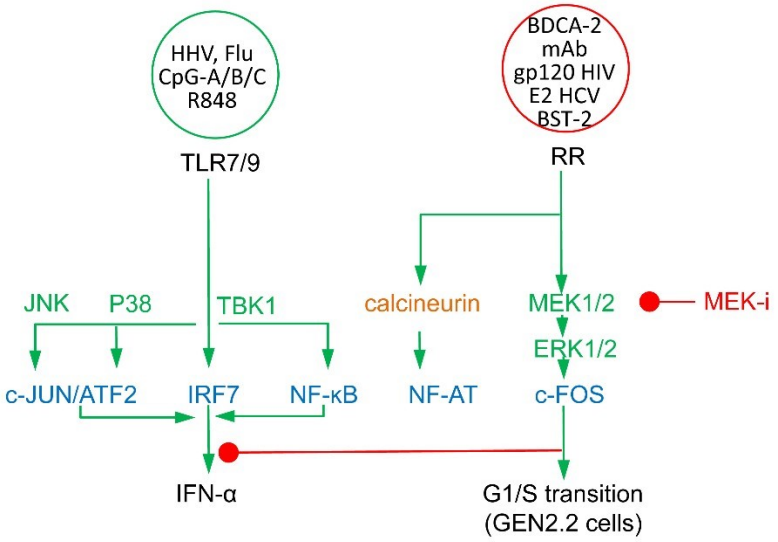


FIGURE S1 | TLR7/9 and BCR-like (RR-triggered) signaling pathways in pDCs. Protein kinases JNK, p38 MAPK, TBK1, MEK1/2 (in green), phosphatase calcineurin (in ochre), and transcription factor NF-κB (in blue) studied in this work are depicted. Transcription factors involved in regulation of expression of IFN-α are shown in blue. Positive signaling pathways are shown by green arrows, negative signaling is in red. IFN-α stimulating TLR7/9 agonists Human herpesviruses (HHV), influenza virus (Flu), CpG-A/B/C, R848 are grouped in a green circle. Agonists of RR (BDCA-2 mAb, gp120 HIV, E2 HCV, BST-2) are grouped in a red circle.

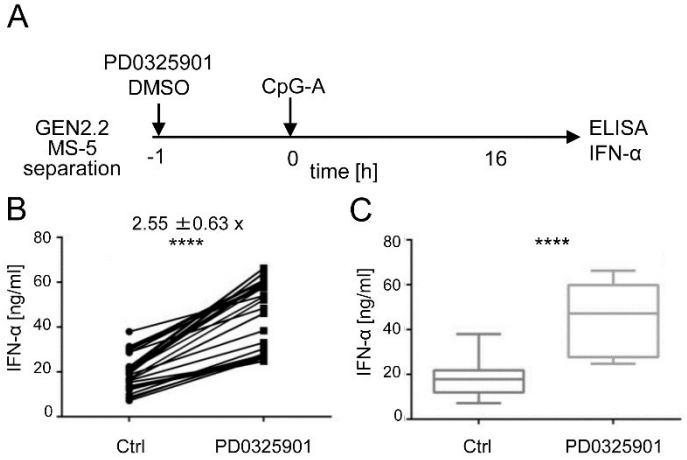


FIGURE S2 | Effect of MEK1/2 inhibitor PD0325901 on the production of IFN-α in CpG-A stimulated GEN2.2 cells. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were incubated or not with 1 µM PD0325901 for 1 h before stimulation with CpG-A. After a 16 h culture, the IFN-α production was determined in the cell-free supernatants by ELISA. **(B)** The data are shown as an aligned dot plot or **(C)** a box-and-whiskers plot (median [interquartile range (IQR)] 17.9 IQR [12.0-21.9] IFN-α (ng/ml), ctrl without PD0325901, 47.2 IQR [27.7-59,8] IFN-α (ng/ml) with PD0325901. N=34, ****, p <0.0001, two-tailed paired Wilcoxon test.

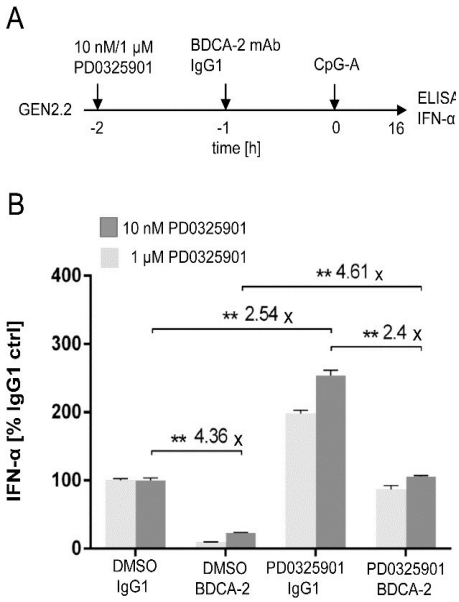


FIGURE S3 | Effect of MEK1/2 inhibitor PD0325901 on the blockade of IFN-α production by ligation of RRs of GEN2.2 cells with BDCA-2 mAb. **(A)** Experimental outline. GEN2.2 cells separated from MS-5 feeder cells were incubated with 10nM or 1 µM PD0325901 for 1 h before stimulation with BDCA-2 mAb and CpG-A. After a 16 h culture, the IFN-α production was determined in the cell-free supernatants by ELISA. **(B)** The IFN-α production was normalized to the level induced in pDCs by CpG-A in the presence of IgG1 and in the absence of the MEK1/2 inhibitor. The data show mean ±SEM of six independent experiments with GEN2.2 cells, **, *p* <0.01 two-tailed Mann-Whitney test.

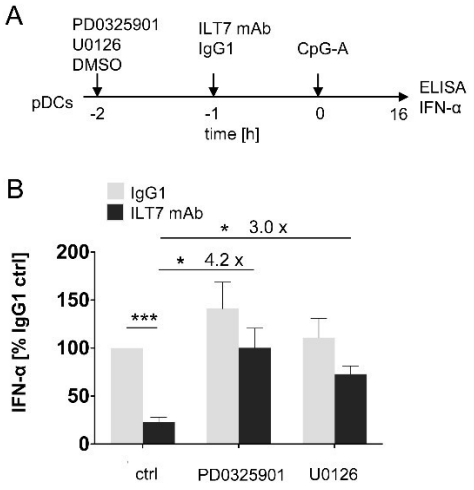


FIGURE S4 | Effect of MEK1/2 inhibitors PD0325901 and U0126 on the blockade of IFN-α production in primary pDCs by ligation of RR ILT-7. **(A)** Experimental outline. Primary pDCs isolated from PBMCs of healthy donors were incubated with 1 µM U0126 (N=5) or 1 µM PD0325901 (N=6) for 1 h before stimulation with ILT-7 antibodies and CpG-A. After a 16 h culture, the IFN-α production was determined in the cell-free supernatants by ELISA. **(B)** The data show mean ±SEM of IFN-α production in five independent experiments with U0126 and six independent experiments with PD0325901 normalized to the level of IFN-α induced in pDCs by CpG-A in the presence of IgG1 and in the absence of the MEK1/2 inhibitor. *, *p* <0.05; *** *p* <0.001; two-tailed paired Wilcoxon test.

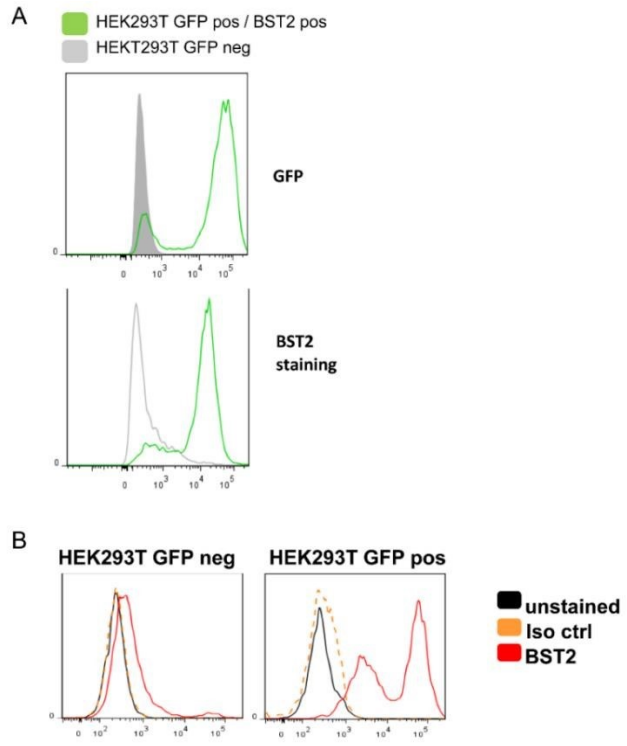


FIGURE S5 | Characteristics of BST2 expressing HEK293T cells. HEK293T cells transduced by lentivirus vector pRRL-BST2-GFP and sorted by GFP marker were analyzed for the expression of GFP and BST2 just after cell sorting **(A)** or after a 2-week culture **(B)**. Both the BST2-transduced (GFP+) cells and mock-transduced HEK293T cells are shown.

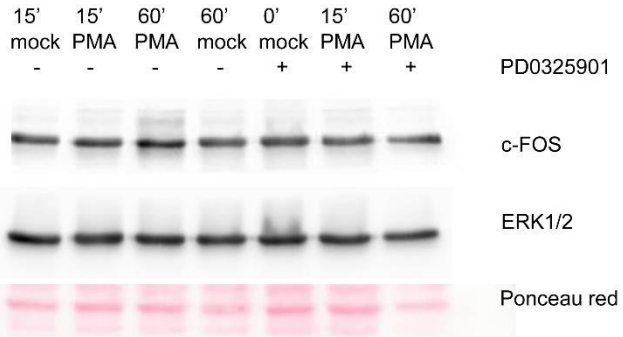


FIGURE S6 | c-FOS and ERK1/2 in PMA-stimulated GEN2.2 cells. GEN2.2 cells separated from MS-5 feeder cells and starved in a serum-free medium for 16 h were pretreated or not with MEK1/2 inhibitor PD0325901 for 1 h and then stimulated with PMA for 0, 15 and 60 min. c-FOS and ERK1/2 were analyzed using Western blotting with rabbit polyclonal c-FOS (sc-52) and ERK1/2 (sc-154) Abs.

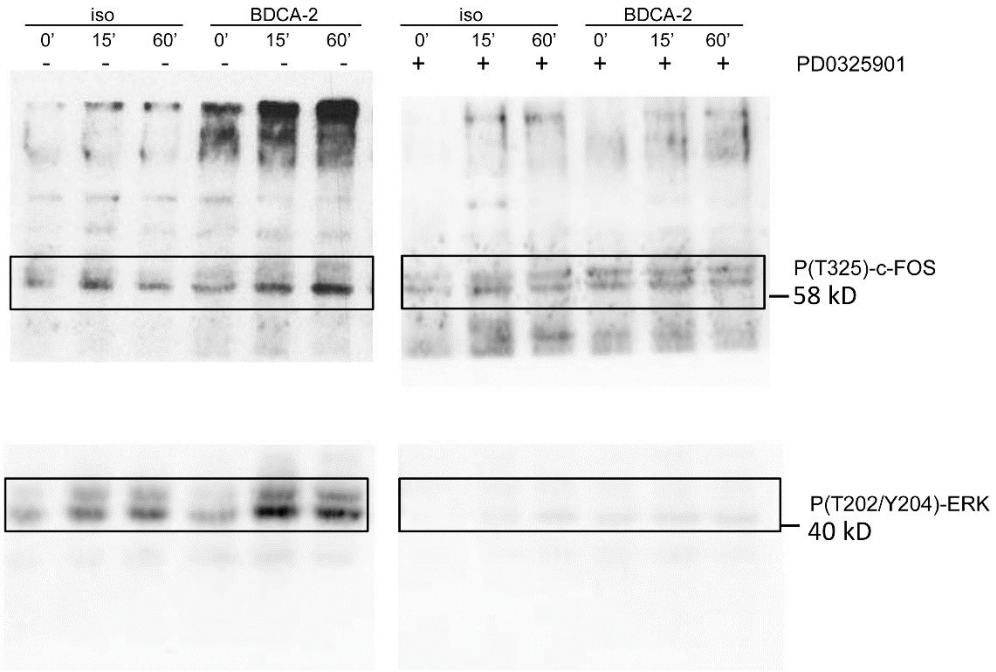
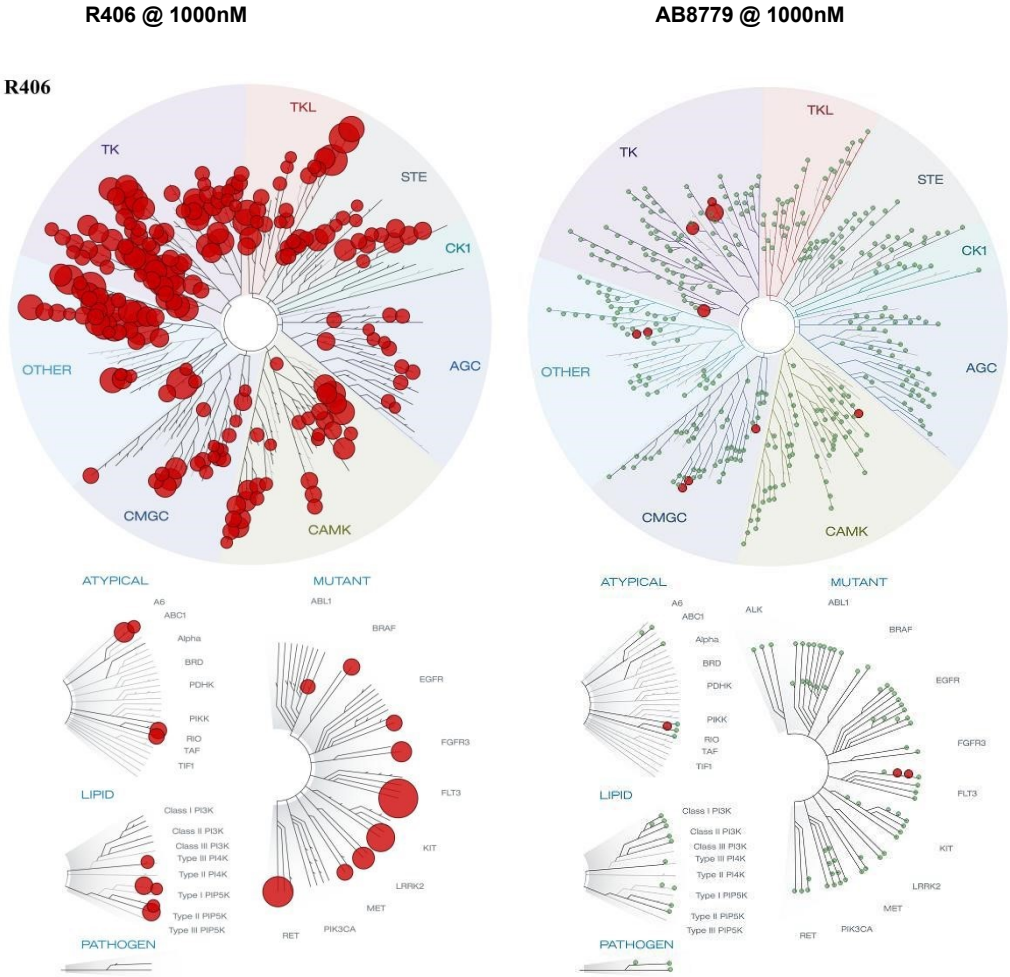


FIGURE S7 | Activation of c-FOS (A) and ERK (B) in GEN2.2 cells stimulated with BDCA-2 mAb – full scans of original gels. GEN2.2 cells separated from MS-5 feeder cells and starved in a serum-free medium for 16 h were pretreated or not with MEK1/2 inhibitor PD0325901 for 1 h and then stimulated with BDCA-2 mAb. **(A)** The activation of c-FOS was evaluated by analysis of c-FOS phosphorylation using Western blotting with the P(T325)-c-FOS antibody. **(B)** The phosphorylation of ERK-1/2 was determined by P(T202/Y204) ERK-1/2. Framed sections of the Western blot are shown in **Figure 10C (in the article)**.



S8 Fig. Treespot™ interaction maps of AB8779 compared to R406 (Fostamatinib). In vitro kinase profiling by DiscoverX. The result of a high-throughput system (KINOMEscan™) for screening of both compounds against large numbers of human kinases (442 kinases) developed by Ambit Biosciences are visualised using a TREEspot™ interaction Maps. Kinases found to bind the compounds are marked with red circles, where larger circles indicate higheraffinity binding. The compounds were screened at the concentration of 1 µM, and results for primary screen binding interactions are reported as percent control (% Ctrl), where lower numbers indicate stronger hits. DMSO is used as a negative control (100% Ctrl) while a high affinity compound is used as a positive control (0% Ctrl). % Ctrl is calculated as follow:

$$\left(\frac{\text{test compound signal - positive control signal}}{\text{negative control signal - positive control signal}} \right) \times 100$$

The S-score of AB8779 tested in this assay is shown in S1 Table. These results clearly show that AB8779 is more specific than fostamatinib (R406).

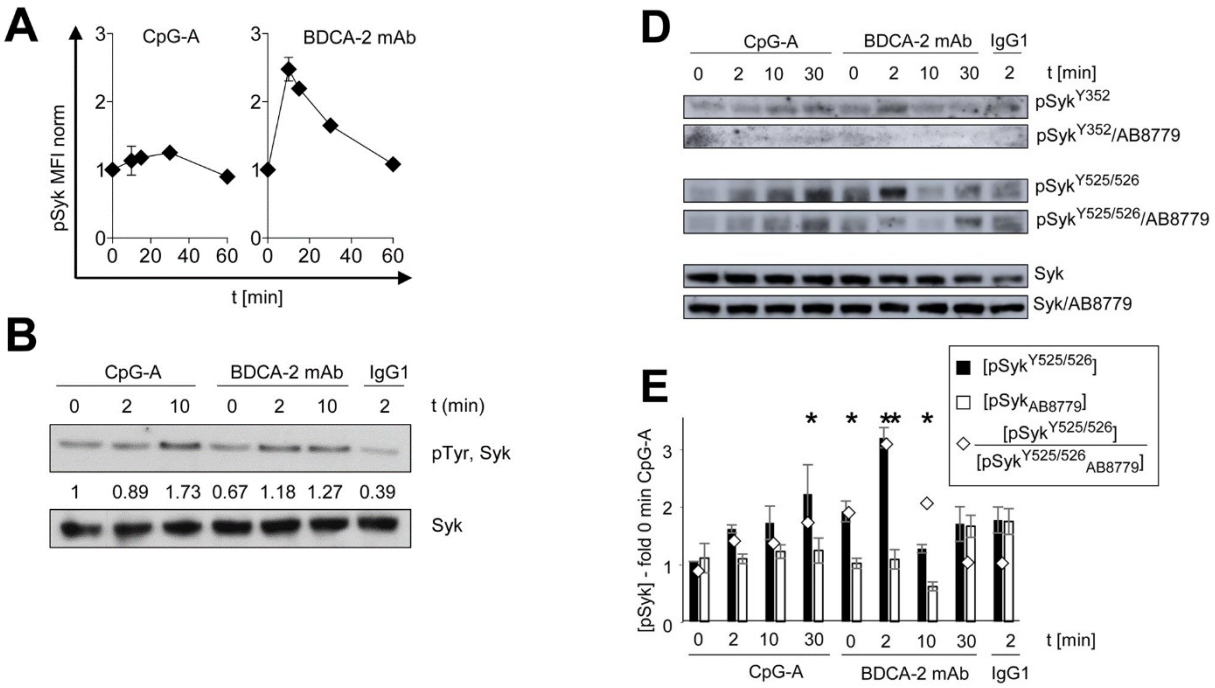


Fig S9. Phosphorylation of Syk in pDCs stimulated by CpG-A or crosslinked with BDCA-2 mAb. (A) Kinetics of phosphorylation of Syk (Y525/526) in the populations of magnetic bead-sorted pDCs exposed to CpG-A or crosslinked with BDCA-2 mAb was followed by flow cytometry (Phosphoflow). The data show means and SEM of three independent experiments with pDCs from different healthy donors. (B) Kinetics of the total Syk phosphorylation in GEN2.2 cells determined by immunoprecipitation of pTyr followed by Western blotting with Syk Ab. Relative quantity of pSyk was determined by densitometry. Total Syk was used as a loading control. (C) Experimental outline. GEN2.2 cells separated from MS-5 feeder cells and serum-starved overnight in RPMI were exposed or not to Syk inhibitor AB8779 for 1.5 h, and then to CpG-A at 4 µg/ml or to BDCA-2 mAb at 10 µg/ml for 20 min at 4°C. BDCA-2-treated cells were crosslinked with F(ab´)2 for 20 min at 4°C, and followed by analysis of phosphorylation of Syk by Western blotting. (D) Kinetics of phosphorylation of Syk Y352 (pSykY352) and Syk Y525/526 (pSykY525/526) in AB8779-treated or non-treated cells stimulated with CpG-A, BDCA-2 mAb or isotype control (IgG1) was followed by western blot. Total Syk was used as a loading control. Representative result of 3 independent experiments. (E) Quantitative densitometric analysis of phosphorylation of Syk Y525/526 (panel D) in the absence (full columns, [pSyk]) and presence (empty columns, [pSykAB8779]) of AB8779 normalized to the total Syk and expressed as fold increase compared to the control (CpG-A 0 min). ^, inhibitory index defined by the ratio of pSyk/pSykAB8779 densities. The data show means and SEM, N = 3. *, p _0.05; **, p <0.01; two-tailed unpaired Student's ttest.

S-score Type	Number of hits/number of non-mutated kinases	S-score
S1	0/395	0
S10	3/395	0.008
S35	11/395	0.028

S1 Table. S-score table for AB8779 tested at 1µM. Selectivity (S)-Score is a quantitative measure of compound selectivity. It is calculated by dividing the number of kinases that compounds bind to by the total number of distinct kinases tested, excluding mutant variants. S(35) = (number of non-mutant kinases with % Ctrl <35)/(number of non-mutant kinases tested), S(10) = (number of non-mutant kinases with % Ctrl <10)/(number of non-mutant kinases tested), S(1) = (number of non-mutant kinases with %Ctrl <1)/(number of non-mutant kinases tested).